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Cloning and analysis of RECK during early Xenopus laevis development

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

CLONING AND ANALYSIS OF RECK DURING EARLY *XENOPUS LAEVIS* DEVELOPMENT

(Spine title: EXPRESSION ANALYSIS OF RECK DURING XENOPUS DEVELOPMENT)

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by

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ABSTRACT

Extracellular matrix (ECM) remodeling is crucial for the development and maintenance of multicellular organisms. Degradation of ECM components occurs through the activity of matrix metalloproteinases (MMPs) and their inhibitors. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) gene encodes a membrane-anchored protein and plays an important role in mediating ECM remodeling by inhibiting MMPs. To date, few *in vivo* studies exist examining RECK during development. The present study focuses on cloning and examining the expression of RECK during early *Xenopus laevis* development. A mature cDNA clone of the *RECK* gene was generated. RT-PCR, *in situ* hybridization, and immunohistochemistry were used to examine the expression of *RECK* during development. *RECK* expression was low during gastrulation but increased during neurulation and into organogenesis. Furthermore, *RECK* was localized to the anterior and dorsal sides of the developing embryo. These results suggest an important role for RECK during late ECM remodeling events, such as neurulation.

Keywords: RECK, Development, ECM remodeling, *Xenopus laevis*, MMPs, TIMPs

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TABLE OF CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

LIST OF APPENDICES

PAGE

LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli
Asn	Asparagine
BSA	Bovine serum albumin
CCR	Corticocytoplasmic rotation
cDNA	Complementary DNA
DAI	Dorsoanterior index
DAPI	4',6-diamidino-2-phenylindole
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
Dvl	Dishevelled
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation factor one alpha
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ERK	External-regulated kinase
ESTs	Expressed sequence tags
GPI	Glycosylphosphatidylinositol
GSK-3 β	Glycogen synthase kinase-3 beta
HCG	Human chorionic gonadotropin
HDAC	Histone deacetylase
Hpf	Hours post fertilization
LB	Luria Bertani
LiCl	Lithium chloride
MEMFA	MOPS, EGTA, magnesium sulfate, formaldehyde
MMP	Matrix metalloproteinase
MMR	Marc's Modified Ringer
MOPS	3-morpholinopropane-1-sulfonic acid
mRNA	Messenger RNA
MT	Membrane-type
NaCl	Sodium chloride
NCBI	National Center for Biological Information
PAX	Paxillin
PBS	Phosphate-buffered saline
PBST	PBS Triton-X
PCR	Polymerase chain reaction
RECK	Reversion-inducing cysteine-rich protein with Kazal motifs
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
Sdp	Sensory deprived
SMART	Simple modular architecture research tool
SSC	Sodium citrate, sodium chloride

st	Stage
TAE	Tris acetate EDTA
TBS	Tris-buffered saline
TBT	TBS, Tween 20, BSA
TIMP	Tissue inhibitor of metalloproteinase
TTw	TBS Tween 20
UV	Ultraviolet

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Extracellular matrix dynamics

The extracellular matrix (ECM) is an extensive network of interacting macromolecules that surrounds cells in tissues. The ECM is primarily composed of fibrous structural proteins such as collagens and fibronectin; however, it also sequesters a variety of important signaling molecules such as cytokines and growth factors. As such, the ECM not only provides structural support to cells in tissue but also regulates cell growth, survival, migration, and differentiation (Nyalendo *et al.*, 2007). Changes in cell-cell and cell-ECM contacts as well as the release of sequestered proteins occur through cleavage and remodeling of the ECM. These processes are crucial for proper development and maintenance of multicellular organisms. Changes and remodeling of the ECM occur through the action of a group of extracellular proteases called matrix metalloproteinases (MMPs) (Takino *et al.*, 1995).

1.2 Matrix Metalloproteinases

MMPs are a large family of zinc-dependent endopeptidases. Twenty-four vertebrate MMPs have been characterized, which together are capable of cleaving all components of the ECM (Visse and Nagase, 2003). MMPs are classified into 2 categories: secreted and membrane-type (MT) MMPs (reviewed in Jiang and Pei, 2003). Most MMPs are secreted, and are released into the ECM as inactive zymogens (pro-MMPs). Once activated through the removal of their prodomain, secreted MMPs can directly cleave ECM proteins as well as release cytokines, growth factors, and other ECM-bound signaling molecules. As their name describes, membrane-type MMPs are bound to the cell surface. MT-MMPs are shuttled to the plasma membrane in an already

active form; their prodomains are removed by the endoprotease furin within the Golgi during secretion. MT-MMPs not only cleave ECM proteins but also activate other secreted pro-MMPs (Wang *et al.*, 2004). Once activated, the family of MMP molecules can collectively cleave all components of the ECM. However, different MMPs have specific substrate specificities, and thus carry out separate functions during ECM remodeling depending on the composition of the ECM (Visse and Nagase, 2003). For example, MMP-2 is a potent gelatinase whose role is to degrade collagen type IV, a major component of the ECM (Jiang and Pei, 2003), whereas MT1-MMP can cleave a number of substrates including collagens I, II, and III, but it is unique in that it has the ability to activate pro-MMP-2 (Visse and Nagase, 2003).

In adults, ECM remodeling is limited. Uncontrolled cleavage of the ECM is often associated with tumor progression and metastasis. Studies have identified a correlation between high MMP activity and tumor progression as a result of excessive ECM degradation (Visse and Nagase, 2003). During development, the extracellular matrix undergoes extensive remodeling to allow processes such as gastrulation and organogenesis to occur. As such, MMPs are highly expressed during development (Alexander *et al.*, 1996). However, aberrant up-regulation of MMPs has been associated with developmental defects and death. For example, Damjanovski *et al.* (2001) generated transgenic frog embryos that overexpressed either MMP-9 or -11 and found that overexpression of these MMPs resulted in embryonic lethality in a dose-dependent manner. Therefore, it is crucial that MMP activity be tightly regulated. Regulation of MMP activity occurs via a number of different inhibitors, including tissue inhibitors of metalloproteinases (TIMPs) (Baker *et al.*, 2004).

1.3 Tissue Inhibitors of Metalloproteinases

TIMPs, endogenous inhibitors of MMPs, are a family of four secreted proteins. TIMP proteins are unique in that they contain structurally and functionally distinct N- and C-terminal domains. The N-terminal domain binds non-covalently to the active site of MMPs and inhibits MMP catalytic activity (Gomis-Ruth *et al.*, 1997). This leaves the C-terminal domain free to carry out other functions, including cell signaling via an ability to bind to distinct cell surface partners (Visse and Nagase, 2003). All four TIMPs are capable of inhibiting the activity of distinct members of the MMP family via their N-terminal domain, however, they function differently at their C-terminal domains. For example, TIMP-1 has been shown to inhibit apoptosis by binding to CD63 and β1 integrin receptors on the cell surface (Chirco *et al.*, 2006), whereas TIMP-2 has been shown to inhibit cell migration by binding to α3β1 integrins on the cell surface (Oh *et al.*, 2004).

A delicate balance between MMP and TIMP levels is crucial for proper development to occur. As such, TIMPs are also expressed during development in order to regulate MMP activity (Alexander *et al.*, 1996). Any shift in balance can disrupt development. For example, overexpression of TIMP-2 or -3 during early frog development both resulted in severe developmental defects (Nieuwesteeg *et al.*, 2012). Until recently, TIMPs were thought to be the main regulators of MMPs, however, a new inhibitor of MMPs was also discovered, known as reversion-inducing cysteine-rich protein with Kazal motifs (RECK).

1.4 RECK structure

RECK was first discovered as a gene that induces flat cell morphology when expressed in *ras*-transformed mouse fibroblasts (Takahashi *et al.*, 1998). The *RECK* gene encodes a GPI-anchored protein with multiple epidermal growth factor (EGF)-like repeats and three serine-protease inhibitor-like domains. These protease inhibitor-like domains are called Kazal motifs as they follow the general amino acid sequence of a Kazal motif (reviewed in Rimphanitchayakit and Tassanakajon, 2010). RECK proteins also contain five repeats of a putative cystine knot motif and five asparagine residues that serve as potential glycosylation sites. The N-terminal region of RECK contains a hydrophobic domain which serves as a signal peptide that is cleaved in the mature protein. The C-terminal region of RECK also contains a hydrophobic domain which serves as a signal for GPI-anchoring to the membrane (Takahashi *et al.*, 1998) (Figure 1).

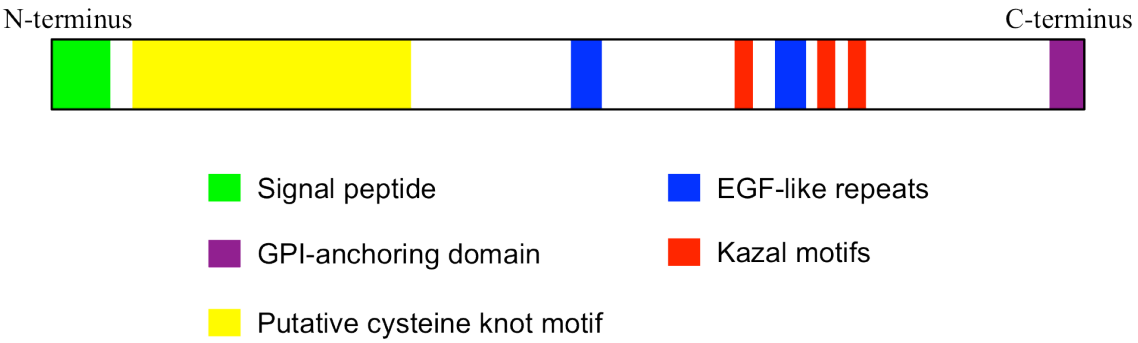
All mammalian RECK proteins that have been cloned to date are highly conserved at the amino acid level and share the same structure as outlined in Figure 1. Mammalian RECK proteins are 971 amino acids in length and weigh approximately 110 kDa (Takahashi *et al.*, 1998). RECK has also been cloned from a variety of non-mammalian vertebrate and invertebrate species and shows evolutionary conservation, however, *Xenopus laevis* RECK has not previously been cloned.

1.5 RECK function *in vitro*

Since its discovery in *ras*-transformed mouse fibroblasts, RECK quickly became known as a tumor suppressor protein. As such, numerous studies have been carried out to determine its anti-invasive properties. *In vitro* studies have shown that RECK can

Figure 1.

Schematic representation of the full length RECK protein. Mammalian RECK is 971 amino acids in length and contains a signal peptide on the N-terminus and a GPI-anchoring domain on the C-terminus. The N-terminal region of RECK contains a putative cysteine knot motif. The middle portion of RECK contains 3 Kazal motifs (serine-protease inhibitor-like domains) and 2 epidermal growth factor (EGF)-like repeats. Modified from Takahashi *et al.* (1998).


N-terminus
C-terminus
Signal peptide
EGF-like repeats
GPI-anchoring domain
Kazal motifs
Putative cysteine knot motif

negatively regulate three MMPs. RECK can inhibit the secretion of MMP-9 and can directly inhibit the enzymatic activities of MMP-2 and MT1-MMP. Interestingly, RECK can also inhibit the expression of MMP-9 (Oh *et al.*, 2001; Takagi *et al.*, 2009; Takahashi *et al.*, 1998) (Figure 2).

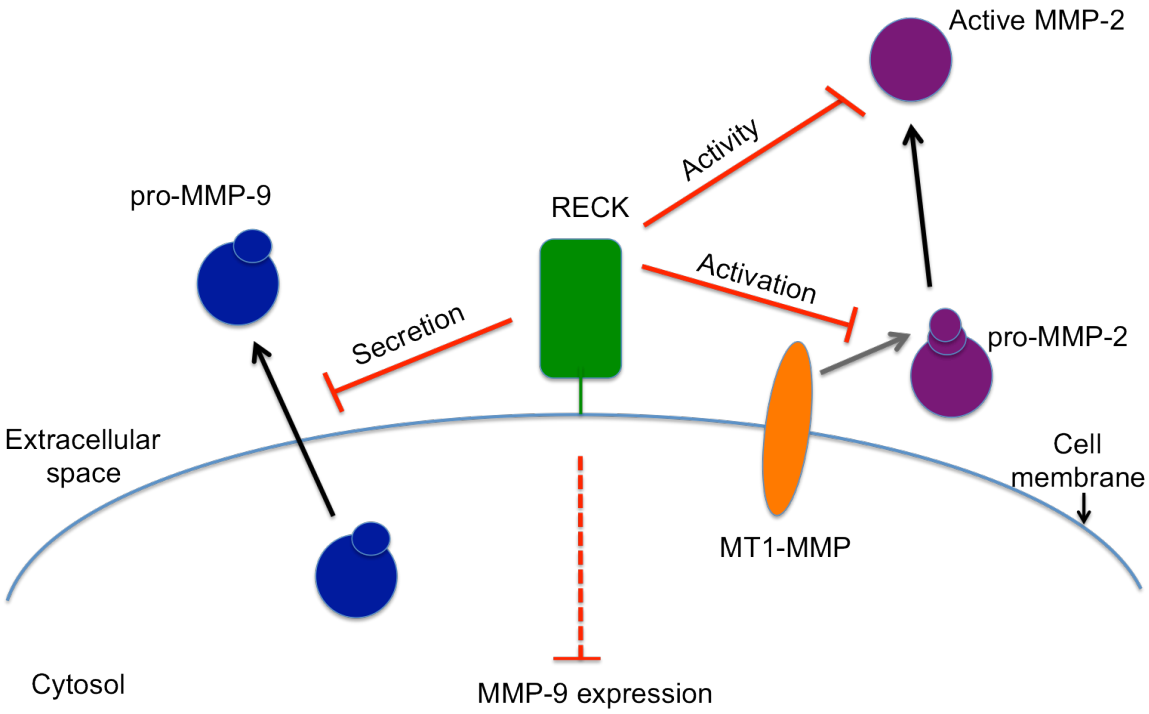
Although *RECK* mRNA is highly expressed in various human tissues, its expression is undetectable in many tumor-derived cell lines (Takahashi *et al.*, 1998). As such, *RECK* expression has been studied in various solid tumors, including breast cancer (Span *et al.*, 2003), lung cancer (Takenaka *et al.*, 2005), pancreatic cancer (Furumoto *et al.*, 2001), and colorectal cancer (Takeuchi *et al.*, 2004). All of these studies arrived at the same conclusion: the more *RECK* is expressed, the better the prognosis is for the patient. Futhermore, studies have shown that when *RECK* expression is restored in tumor cell lines invasiveness decreases (Oh *et al.*, 2001; Takahashi *et al.*, 1998; Simizu *et al.*, 2005; Chang *et al.*, 2008). Takahashi *et al.* (1998) generated stable transfectants of *RECK*-expressing fibrosarcoma cell lines and found that invasiveness decreased in *RECK*-expressing cells. Moreover, Oh *et al.* (2001) found that re-expression of *RECK* in HT1080 cells tumors in mice repressed tumor angiogenesis. Altogether, these results classify RECK as a tumor suppressor protein that can decrease invasiveness by inhibiting MMP activity.

1.6 Regulation of RECK

Since its discovery, the majority of research characterizing RECK focuses on its ability to suppress tumor cell invasion. However, more studies have arisen regarding how RECK is regulated. *RECK* was first discovered in *ras*-transformed mouse

Figure 2.

Effects of RECK on MMPs. RECK is bound to the membrane through GPI-anchoring. RECK inhibits the secretion of pro-MMP-9 and directly inhibits the enzymatic activity of MT1-MMP and MMP-2, as well as inhibiting the expression of MMP-9. MMP-2 and -9 (gelatinases) are potent enzymes whose functions are associated with important developmental events and diseases. MT1-MMP is an important cell surface MMP whose role, amongst others, is to activate MMP-2. Modified from Oh *et al.* (2001).


Active MMP-2
Activity
pro-MMP-9
RECK
Activation
Secretion
pro-MMP-2
Extracellular
space
Cell
membrane
MT1-MMP
Cytosol
MMP-9 expression

fibroblasts. This suggests regulation by oncogenic signaling (Takahashi *et al.*, 1998). More recently, Chang *et al.* (2004) revealed that RECK is regulated at the transcriptional level through Sp1-binding sites (GC boxes) located downstream of the transcriptional start site. This binding site serves as a *cis*-element and is involved in repressing *RECK* expression. Phosphorylation of Sp1 occurs by external signal-regulated kinase (ERK). Phosphorylated Sp1 and histone deacetylase (HDAC) 1 bind to the Sp1 binding site and repress *RECK* expression. Furthermore, Liu *et al.* (2002) revealed that Trichostatin A, a histone deacetylase inhibitor known to suppress tumor invasion, was shown to activate the RECK promoter and cause a decrease in secreted MMP-2.

RECK has also been shown to be regulated at the post-translational level. As mentioned previously, RECK contains five potential glycosylation sites. Simizu *et al.* (2005) identified that three of the five glycosylated asparagine (Asn) residues of RECK are required to suppress tumor cell invasion. Simizu *et al.* (2005) generated *RECK* mutants in which they replaced asparagine residues with glutamine residues. Mutant and wild-type *RECK* genes were transfected into HT1080 cells and *MMP-2* and *-9* expression were subsequently assayed. Results indicated that glycosylation of RECK Asn^{297} was required to inhibit MMP-9 secretion and glycosylation of RECK Asn^{352} was required to inhibit the activation of pro-MMP-2. Furthermore, Simizu *et al.* (2005) correlated glycosylation of RECK with tumor cell invasion and found that glycosylation of RECK at Asn^{86}, Asn^{297}, and Asn^{352} was required to suppress HT1080 cell invasion.

Interestingly, RECK has also been shown to be regulated by another key player of ECM remodeling. TIMP-2 is a well-characterized MMP inhibitor. As described, TIMP-2 can bind directly to MMPs in the ECM and inhibit their function. Additionally, TIMP-

2 can also indirectly up-regulate RECK, which in turn inhibits MMPs. Specifically, TIMP-2 binds to α3β1 integrins on the surface of human endothelial cells, inactivates Src kinase through changes in paxillin (PAX) phosphorylation, and activates the small GTPase Rap1, which ultimately results in up-regulation of RECK (Oh *et al.*, 2004) (Figure 3).

1.7 *Xenopus laevis* as a model system

The model organism used in this study is *Xenopus laevis*, the African clawed frog. *Xenopus laevis* is an ideal developmental model organism with which complex biological processes can be investigated. Hundreds of embryos can be obtained from a single fertilization, development occurs rapidly, and embryos are large and easy to manipulate. Furthermore, fate maps and cell signaling cascades have been well characterized in *X. laevis,* making it an ideal model to study novel gene and protein functions during development.

Xenopus laevis has long been used as a developmental model and undergoes typical embryological processes such as gastrulation (stage 10), formation and closure of the neural tube (stages 14 and 20, respectively), and organogenesis (stage 28 to 40) (Figure 4). All of these processes involve large-scale cell movements and therefore require extensive remodeling of the ECM. As previously mentioned, MMPs are highly expressed during development to regulate remodeling of the ECM in a temporal and tissue specific manner (Alexander *et al.*, 1996). However, a delicate balance between the levels of MMPs and their inhibitors is crucial for proper development to occur. Since RECK functions by inhibiting MMPs, its presence during development would suggest

Figure 3.

TIMP-2 induction of *RECK* expression. TIMP-2 binds to α3β1 integrins on the cell surface, causing inactivation of Src through changes in paxillin (PAX) phosphorylation. Alteration in PAX phosphorylation mediates the activation of Rap1, ultimately resulting in increased expression of RECK, a membrane-anchored MMP inhibitor. Enhanced *RECK* expression is associated with inhibition of cell migration (Adapted from Stetler-Stevenson, 2008).

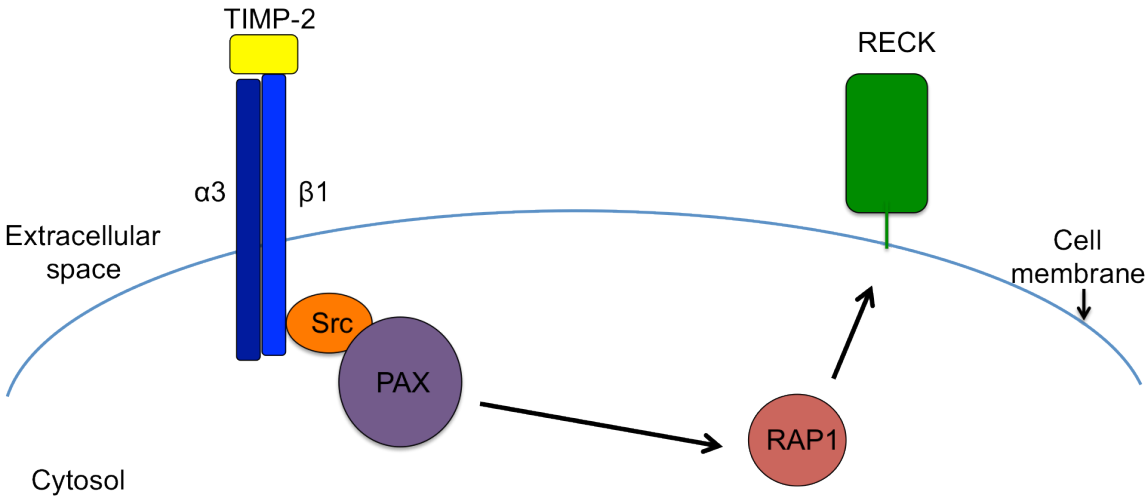
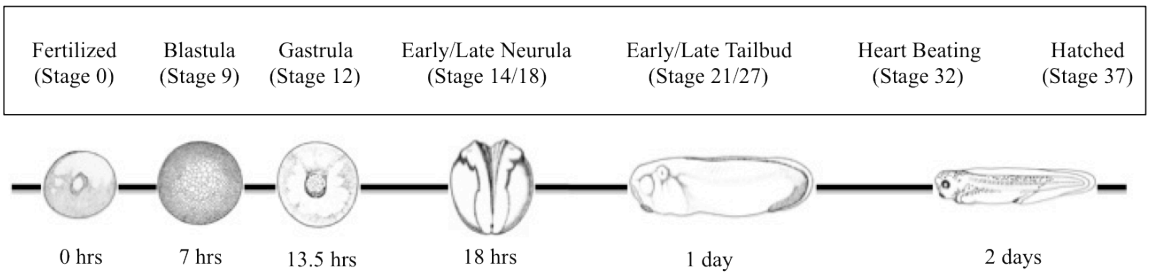

TIMP-2
RECK
α3
β1
Extracellular
space
Cell
membrane
Src
PAX
RAP1
Cytosol

Figure 4.

Early developmental stages of *X. laevis*. Development starts with a fertilized single-celled egg. Zygotic transcription begins at stage 9. Gastrulation occurs at stage 10, forming the three germ layers: endoderm, ectoderm, and mesoderm as well as embryonic axes. Neurulation begins at stage 14, which forms the neural tube by stage 20. Organogenesis is predominant at stage 28 and beyond during which the majority of the internal organs begin to develop. Embryos become feeding tadpoles at about stage 40. Modified from Nieuwkoop and Faber (1994).


Fertilized (Stage 0)
Blastula (Stage 9)
Gastrula (Stage 12)
Early/Late Neurula (Stage 14/18)
Early/Late Tailbud (Stage 21/27)
Heart Beating (Stage 32)
Hatched (Stage 37)
0 hrs
7 hrs
13.5 hrs
18 hrs
1 day
2 days

that it plays important developmental roles as well.

1.8 RECKing development

Since its discovery, most research characterizing RECK has been done *in vitro* in tumor-derived cell lines. To date, few *in vivo* studies exist examining RECK function during development. Oh *et al.* (2001) revealed that *RECK*-deficient mouse embryos died at embryonic day 10.5 because of abnormal vascularization, disrupted organogenesis, abdominal hemorrhaging, and smaller body size. Histological examination of tissues in *RECK*-deficient embryos indicated abnormally large and deformed blood vessels. The results implicated a role for RECK in vascular development, probably due to the ability of RECK to inhibit MMPs (Oh *et al.*, 2001; Chandana, *et al.*, 2010).

A more recent study analyzed RECK function during early zebrafish development and revealed the importance of RECK during the development of dorsal root ganglia (DRG). DRG are derived from neural crest cells and are comprised of sensory neurons along with their associated glia. Prendergast *et al.* (2012) examined zebrafish mutants unable to form DRG, termed sensory deprived (*sdp*), and discovered that this phenotype resulted due to a mutation in the *RECK* gene. When *RECK* wasn't expressed, the DRG failed to form. Furthermore, *RECK* knockdown in zebrafish embryos also resulted in impairment of vascular integrity (Prendergast *et al.*, 2012). These results support the previous mouse knockout studies indicating RECK was involved in vascular development, and also reveal the importance of RECK in the development of DRG. Thus, RECK embryological data show that RECK plays a role associated with DRG formation and vascular development, processes that involve cell migration and ECM

remodeling. However, a more comprehensive characterization of RECK during development is warranted. Given its association with cell migration and ECM remodeling, embryonic processes such as gastrulation and neurulation require further examination. Thus, a simple way to determine a possible role for RECK is by disrupting axis formation in the developing embryo.

1.9 Axis perturbation in *X. laevis* embryos

Axis specific signaling pathways are well understood in *X. laevis* development. During development, the Wnt signaling pathway plays an important role in dorso-ventral patterning of the embryo (Reviewed in Sokol 1999). The Wnt pathway is highly conserved and consists of a large number of proteins that altogether control cell-cell communication in multicellular organisms. In the absence of Wnt, β-catenin is targeted for degradation through association with a destruction complex (Axin, Adenomatous Polyposis Coli (APC), and glycogen synthase kinase-3 β (GSK-3 β)) (Hart *et al.*, 1998; Yost *et al.*, 1996; Aberle *et al.*, 1997). However, when Wnt is present, it binds to cell surface receptors and induces a signaling cascade that activates Dishevelled (Dvl). Dvl recruits Axin away from the destruction complex, inhibiting the degradation pathway and allowing β-catenin to accumulate in the cytosol and nucleus (Yamamoto *et al.,* 1999).

During embryonic development, the Wnt pathway is turned on in areas of the embryo fated to become dorsal. However, if this pathway is turned on in other areas of the embryo not fated to become dorsal, then dorso-ventral patterning is disrupted and dorsalization is induced. Dorsalization can be monitored by visualization of increases in the size of dorsal structures, such as the notochord and eyes, and with increased levels of

dorsal genes, such as chordin. On the other hand, if the Wnt pathway is turned off in an area of the embryo fated to become dorsal, then ventralization is induced. Thus, with ventralized embryos, there will be increased ventral structures and genes, and a concomitant decrease in dorsal structures and genes.

When examining the complex roles a molecule may play during development, a simple approach is to perturb axis formation in early *X. laevis* embryos. This method aids in dissecting out functions of genes in early development. There are a variety of physical and chemical treatments that can be used to turn on/off the Wnt signaling pathway and thus perturb axis formation. By doing so, a gene of interest can be studied to determine if its expression-associated effects are altered by the dorsalization/ventralization of the embryo. Lithium chloride (LiCl) can be used on early frog embryos to induce dorsalization (Kao and Elinson, 1988), whereas ultraviolet light (UV) can be used to induce ventralization (Scharf and Gerhart, 1983).

1.10 Dorsalization via lithium chloride treatment

LiCl induces dorsalization due to its effect on the Wnt pathway. Lithium inhibits GSK-3 β, a member of the destruction complex. This activates the Wnt pathway and prevents β-catenin from being degraded. Accumulation of β-catenin in areas not fated to become dorsal disrupts dorso-ventral axis patterning and results in dorsalized embryos (Klein and Melton, 1996). Thus, if the whole embryo is treated with LiCl, all tissues will be dorsalized. The higher the dosage of LiCl, the more dorsalized the embryo will become.

1.11 Ventralization via ultraviolet light treatment

Dorso-ventral polarity is established during the first cell cycle in the forming frog zygote. Following fertilization of an egg, a 30° rotation of the cortical cytoplasm relative to the cytoplasmic core, known as corticocytoplasmic rotation (CCR), occurs. This process is mediated by a microtubule-associated motor and takes place on microtubules present on the vegetal side of the embryo (Elinson and Rowning, 1988). Rotation results in the protection from degradation and accumulation of the dorsal determinant β-catenin on the future dorsal side of the embryo. Thus, β-catenin localization begins the establishment of the dorso-ventral axis (Heasman 1997; Harland and Gerhart, 1997). When embryos are exposed to UV irradiation prior to completion of the first cell cycle, UV light depolarizes microtubules and prevents CCR. Without CCR, β-catenin is degraded everywhere and is not present to establish dorsal tissues. Thus, ventralization of embryos occurs.

1.12 Dorsoanterior index scale

Sensitivity to axis perturbation treatments varies from embryo to embryo, causing a range of altered phenotypes to develop. In order to characterize axis-perturbed embryos, a Dorsoanterior index scale (DAI) ranging from 1 to 10 was created by Kao and Elinson in 1998 and is currently the accepted standard. A DAI of 0 is assigned to embryos that are completely ventralized and thus lack dorsoanterior structures. Embryos assigned a DAI of 0 are comprised largely of yolk and have no head or tail present. A DAI of 10 is assigned to embryos that are completely dorsalized and thus have very enhanced dorsoanterior structures. Embryos assigned a DAI of 10 have exaggerated

heads with bands of retinal pigment and cement glands. Normal embryos fall in the middle of the scale and are assigned a DAI of 5.

1.13 Hypothesis and investigation plan

RECK has been implicated to be an important regulator of ECM remodeling. Furthermore, during mouse and zebrafish development, RECK has been shown to play an important role during vascular development and neurulation. **I hypothesize that RECK will be differentially expressed throughout *X. laevis* development to regulate ECM remodeling.**

To test this hypothesis, I cloned and sequenced the mature coding region of *X. laevis RECK*. Subsequently, I examined the expression pattern of *RECK* during embryogenesis both temporally using semi-quantitative PCR and spatially using whole mount *in situ* hybridization and immunohistochemistry. Furthermore, I perturbed axis formation in embryos by inducing either dorsalization or ventralization and examined if *RECK* transcript levels changed as a result. Since few *in vivo* studies exist examining *RECK* expression during development, my project is the first detailed comprehensive characterization of RECK during *X. laevis* development. Overall, this project provides a better understanding of RECK expression during development, and improves our understanding of its role as a regulator of ECM remodeling.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal care and rearing

Adult male and female *X. laevis* were purchased from Xenopus 1, Inc. (Dexter, MI) and reared at room temperature in freshwater tanks on a 12-hour light/dark cycle. Fertilizations were carried out by injecting adult females with 300 μL of human chorionic gonadotropin (HCG; Chorulon, Whitby, ON) 18 hours prior to ovulation. Adult males were sacrificed and dissected to remove the testes, which were stored for up to 1 week in 1x Marc's Modified Ringer solution (MMR; 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2 mM $NaHCO_3$, 10 mM HEPES, pH 7.4) at 4°C.

For fertilization, a small portion of a testis was minced and combined with eggs from an ovulating female in a Petri dish with 1 mL of 1x MMR. After 2 minutes, the dish was flooded with 0.1x MMR. Following cortical rotation (30 minutes post fertilization), embryos were de-jellied in a 3% cysteine solution (L-cysteine free base in 0.1x MMR, pH 8.0) and then maintained in 0.1x MMR at room temperature until the desired developmental stage was reached. Developing embryos were staged as previously described (Nieuwkoop and Faber, 1994). All procedures were in compliance with the animal care standards set by The Canadian Council on Animal Care.

2.2 Polymerase chain reaction (PCR) amplification of the full length coding region of *X. laevis RECK*

In order to examine *RECK* expression during early *X. laevis* development, the *RECK* gene first needed to be cloned and sequenced. However, *X. laevis* sequences are poorly annotated due to their pseudotetraploid genome. As such, the online database of *Xenopus tropicalis* was used instead as this species is evolutionarily related to *X. laevis*.

Based on the *X. tropicalis* RECK sequence obtained online (accession number: XM_002938937.1), forward and reverse primers were engineered flanking the mature coding region of the *RECK* gene (Table 1). Primers were also generated flanking a highly conserved region of RECK (the three Kazal motif domains) for subsequent experiments (Table 1). Total RNA was isolated from adult *X. laevis* intestinal tissue (as mouse intestinal tissue is known to express RECK) using an RNeasy Kit (QIAGEN, Germantown, MD) according to manufacturer's instructions. First strand cDNA synthesis and PCR were performed on total RNA using qScript™ Reverse Transcriptase (Quanta Biosciences, Gaithersburg, MD) and Kapa Hi-Fi Taq PCR Kit (Kapa Biosystems, Woburn, MA) according to manufacturer's instructions. The amplification program consisted of 94°C for 2 minutes, then cycling of 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 2 minutes for 32 cycles, followed by 68°C for 10 minutes. PCR products were visualized on a 1% TAE (40 mM Tris acetate, 1 mM EDTA) agarose gel and bands corresponding to the expected amplicon size were excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer's instructions. The purified amplicons were cloned into the pGEM-T vector (Promega, Madison, WI) and transformed into One Shot TOP 10 Chemically Competent *E. coli* cells (Life Technologies, Burlington, ON) and plated on Fastmedia Luria Bertani (LB) Agar Amp (Thermo Scientific, Waltham, MA). The plates were incubated at 37°C for 24 hours. Colonies were picked, resuspended in 5 mL LB broth, and grown overnight at 37°C. Plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN). Identity of the PCR products was confirmed by sequencing carried out at the DNA Sequencing Facility at Robarts Research Institute (London, ON).

Table 1.

Oligonucleotide primers used for cloning the mature open reading frame of *X. laevis RECK*.

Amplicon	Primer Sequence	Amplicon Size (Base Pairs)	Nucleotide Position Numbers*
Full length RECK	$^{5'}$ATGTGTCGTGATGTATGTGA$^{3'}$	2, 532	205-224
	$^{3'}$CTAGTGTGTGTCCGGCTTGT$^{5'}$		2819-2838
Kazal motif domains	$^{5'}$GGATGTTTACAGGTCTACCC$^{3'}$	494	1904-1923
	$^{3'}$ATGGACACTGCCAGGATGTT$^{5'}$		2378-2397

*Nucleotide position numbers refer to the *X. tropicalis* RECK sequence (Accession number: XM_002938937.1)

2.3. Sequence analysis

To confirm its identity, *X. laevis* RECK sequence obtained in this study was compared to RECK sequences from a variety of other species. Sequence analysis was carried out using RECK protein sequences obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/pubmed/) as well as the predicted *X. laevis* RECK protein sequence. RECK sequences along with the accession numbers used for comparison were from the following species: *Homo sapiens* (NP_066934), *Canis lupis familiaris* (NP_001002985), *Mus musculus* (NP_057887), *Gallus gallus* (XP_418897), *Xenopus laevis* (cloned in this study), *Oreochromis niloticus* (XP_003457562), and *Drosophila melanogaster* (NP_648733). Full-length RECK amino acid sequences were aligned using ClustalW2 Multiple Sequence Alignment web software at the European Bioinformatics Institute (EBI) site at (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and given a score based on similarity. The structural domains of RECK were determined using the SMART web software (http://smart.embl-heidelberg.de/). All analyses were performed using default program settings.

2.4 Reverse transcriptase PCR (RT-PCR)

To investigate temporal expression of *RECK* during early *X. laevis* development, semi-quantitative PCR was performed. Total RNA was isolated from embryos at stages 10, 18, 24, 32, 37, and 40 using an RNeasy Kit (QIAGEN). First-strand cDNA synthesis and PCR were performed on total RNA using qScript^{TM}Reverse Transcriptase (Quanta Biosciences) and KapaTaq (Kapa Biosystems) according to manufacturer's instructions. The amplification program consisted of 95°C for 2 minutes, then cycling of 95°C for 30

seconds, 60°C for 30 seconds, and 72°C for 30 seconds for 30 cycles (in the exponential phase). The gene specific primers used for RT-PCR were as listed (Table 2). PCR products were visualized on a 1% TAE agarose gel and quantified against elongation factor one alpha (EF1α) to calculate a percentage of expression (gene of interest over EF1α) with Quantity One software (version 4.4.1 Bio-Rad Laboratories, Hercules, CA). All quantified PCRs were repeated with three biological replicates.

2.5 Synthesis of DIG-labeled RNA probes and whole mount *in situ* hybridization

To investigate spatial expression of *RECK* during early *X. laevis* development, whole mount *in situ* hybridization was performed. Digoxigenin (DIG)-labeled anti-sense RNA probes were generated from the Kazal motifs cDNA clone (494 base pairs; Table 1) in pGEM-T vector (Promega) using SP6 RNA Polymerase and the DIG RNA Labeling Kit (SP6/T7; Roche, Laval, QC) according to the manufacturer's instructions. DIG-labeled *RECK* RNA probes were purified using an RNeasy® Kit (QIAGEN). DIG-labeled *cardiac troponin 1* RNA probes were generously provided by Dr. Tom Drysdale (Western University). Embryos from stages 23, 28, 35, and 38 were fixed in MEMFA (0.1M MOPS, 2mM EGTA, 1mM magnesium sulfate, 3.7% formaldehyde) buffer and stored in 100% methanol at -20°C. For hybridization, embryos were rehydrated gradually with methanol and TTw (1x TBS 0.1% Tween-20 (Sigma, St. Louis, MO)) by 5 minute incubations in 100% methanol, 75% methanol + 25% TTw, 50% methanol + 50% TTw, 25% methanol + 75% TTw, and 100% TTw. They were then washed twice, 5 minutes each, in TTw. Unless otherwise stated, all steps were done at room temperature with a nutator. Embryos were washed twice, 5 minutes each, in triethanolamine (Sigma).

Table 2.

Oligonucleotide primers used for semi-quantitative PCR to characterize levels of developmental gene expression in *X. laevis.*

Gene	Primer Sequence	Amplicon Size (Base Pairs)	Nucleotide Position Numbers*
Chordin	5'AACTGCCAGGACTGGATGGT3'	190	3201-3220
	3'GGCAGGATTTAGAGTTGCTTC5'		3391-3411
EF1-α	5'CAGATTGGTGCTGGATATGC3'	268	1096-1115
	3'ACTGCCTTGATGACTCCTAG5'		1345-1364
RECK	5'GGATGTTTACAGGTCTACCC3'	147	1724-1744
	3'ATCTTTGGAGGAACAGAGCC5'		1851-1871
TIMP-2	5'CCAAAGCCGTTACTGGAAAA3'	218	261-280
	3'CATCACCATCGGCTTTACCT5'		460-479

*Nucleotide position numbers refer to the following *X. laevis* sequences: chordin (BC077767.1), EF1-α (M25504.1), TIMP-2 (NM_001094279.1)

Acetic anhydride was added to triethanolamine in a 1:400 dilution and embryos were again washed twice, 5 minutes each. This step was monitored carefully, as embryos will degrade the next day if left too long. Embryos were then washed five times, 5 minutes each, in TTw, followed by a 10 minute wash in 50% RNA hybridization buffer (50% formamide (Sigma), 5x SSC (45mM sodium citrate, 750mM NaCl, pH 7.0), 1 mg/mL Torula RNA, 1x Denhardt's, 100 µg/mL Heparin, 0.1% Tween 20, 5 mM EDTA) + 50% TTw. Embryos were allowed to settle in RNA hybridization buffer for 10 minutes, then fresh RNA hybridization buffer was added and embryos were prehybridized for 2 hours at 65°C. The prehybridization buffer was replaced with fresh RNA hybridization buffer containing the probe (5-10 µg/mL) and embryos were incubated overnight at 65°C. Probes were saved the next day for future use.

RNA Hybridization buffer was gradually washed away with 10 minute washes of 100% RNA hybridization buffer, 50% RNA hybridization buffer + 50% 2x SSC, and 25% RNA hybridization buffer + 75% 2x SSC all done at 65°C. Embryos were washed in 2x SSC twice for 30 minutes each at 37°C, followed by three washes, 45 minutes each, in 0.2x SSC at 65°C. Embryos were washed for 10 minutes in TTw, followed by a 10 minute wash in TBT (1x TBS + 0.1% Tween 20 + 1% BSA). Embryos were blocked in 20% heat-treated sheep serum in TBT for 1 hour. Alkaline phosphatase conjugated DIG antibody (Roche) was added to the blocking solution at a 1:5000 dilution and embryos were incubated overnight at 4°C.

To remove antibody, embryos were washed 12 times, 30 minutes each, in TBT, and then washed for 10 minutes in alkaline phosphatase (100 mM Tris (pH 9.5), 50 mM magnesium chloride, 100 mM sodium chloride, 0.1% Tween 20) buffer. BM purple AP

substrate, precipitating (NBT/BCIP ready to use) (Roche) was added to the alkaline phosphatase buffer and left until desired intensity was reached. The reaction was stopped by gradually dehydrating with methanol and TTw by 5 minute incubations with 25% methanol + 75% TTw, 50% methanol + 50% TTw, 75% methanol + 25% TTw, and 100% methanol. Embryos were rehydrated again in methanol and TTw by 5 minute incubations in 75% methanol + 25% TTw, 50% methanol + 50% TTw, and 25% methanol + 75% TTw, and fixed for 20 minutes in MEMFA. Embryos were washed three times, 5 minutes each, in TTw and then bleached for 4 hours in a bleaching solution (5% formamide (Sigma), 2.5% 20x SSC, 1% hydrogen peroxide in dH_2O). To remove bleaching solution, embryos were washed 3 times, 5 minutes each, in TTw. For imaging, embryos were cleared in a 2:1 mixture of benzyl benzoate:benzyl alcohol to observe internal structures. Embryos were visualized using an Olympus SZX9 microscope system and images were captured using a Nikon Coolpix 990 camera. Images were processed using Adobe Photoshop CS5 Extended, version 12.0.

2.6 Immunohistochemistry and fluorescence microscopy

To investigate the spatial expression of RECK proteins during early *X. laevis* development, immunohistochemistry was performed. Embryos at stage 25 were fixed in 3.7% paraformaldehyde in 1x PBS solution for 2 hours at room temperature and gradually dehydrated in ethanol. Fixed embryos were sent to the Molecular Pathology Core Facility at Robarts Research Institute (London, ON) for sectioning. Slides were washed in xylene for 15 minutes to deparaffinize sections. Embryo sections were gradually rehydrated with ethanol and 1x PBST (1x phosphate-buffered saline + 0.1% Triton-X (Sigma)) by 5 minute incubations in 100% ethanol, 90% ethanol + 10% 1x

PBST, 80% ethanol + 20% 1x PBST, and 100% 1x PBST. Sections were blocked with 10% goat serum in 1x PBST for 45 minutes. Primary antibody (rabbit anti-RECK (Genetex, Irvine, CA) and rabbit anti-β-catenin (Life Technologies)) at a 1:50 dilution was added to 2% BSA in 1x PBST and sections were incubated for 2 hours. Sections were washed with 1x PBST for 5 minutes and a fluorescent-labeled secondary antibody (Alexa Fluor Goat Anti-Rabbit IgG) (Life Technologies) was added to 2% BSA in 1x PBST at a 1:200 dilution and sections were incubated for 1 hour. Sections were washed in 1x PBST for 5 minutes and treated with 4', 6-diamidino-2-phenylindole (DAPI) (Life Technologies) in 1x PBST at a dilution of 1:4000 for 5 minutes, followed by a wash with 1x PBST for 5 minutes. Sections were rinsed with double distilled H_2O and mounted using 50 µL of ProLong Gold antifade reagent (Life Technologies). The slides were visualized using a Zeiss LSM 5 Duo confocal microscope at the Biotron (Western University).

2.7 Lithium chloride treatment of early *X. laevis* embryos

To investigate changes in *RECK* expression as a result of altering dorso-ventral axis patterning in *X. laevis* embryos, dorsalization was induced. Embryos at stage 6 (32-cell stage) were treated with 0.3M lithium chloride in 0.1x MMR for 10 minutes, rinsed 3 times with 0.1x MMR, and transferred to a fresh petri dish containing 0.1x MMR. Embryos were maintained in 0.1x MMR until the desired developmental stage was reached. Phenotypes were examined using the Dorsoanterior Index (DAI) Scale (Kao and Elinson, 1988). Total RNA was isolated from treated and non-treated embryos at stages 20, 30, and 40 and RT-PCR was performed as previously described. The experiment was repeated 3 times.

2.8 Ultraviolet light treatment of early *X. laevis* embryos

To investigate changes in *RECK* expression as a result of altering dorso-ventral axis patterning in *X. laevis* embryos, ventralization was induced. Prior to cortical rotation, embryos at stage 1 (1-cell stage) were exposed to 254 nm UV light at 2500 $\mu W/s/cm^2$ for 3 minutes using a Benchtop 3UV^{TM} Transilluminator (UVP, Upland, CA). Embryos were maintained in 0.1x MMR until the desired developmental stage was reached. Phenotypes were assessed using the DAI Scale (Kao and Elinson, 1988). Total RNA was isolated from treated and non-treated embryos at stages 20, 30, and 40 and RT-PCR was performed as previously described. The experiment was repeated 3 times.

CHAPTER 3

RESULTS

3.1 Cloning of *X. laevis RECK*

The mature RECK protein sequence (lacking the signal peptide and GPI-anchoring signal) was chosen to be cloned in this study as preliminary studies show low conservation of the N- and C-terminal domains of RECK proteins among species. Furthermore, the *X. laevis* database is not fully annotated. As such, primers were chosen flanking known functional domains of *RECK*. The mature coding region of *X. laevis RECK* was amplified from adult intestinal tissue by PCR using primers designed against the *X. tropicalis RECK* sequence obtained online (see Materials and Methods for the accession number and primer sequences). Derived *X. laevis RECK* was amplified, sequenced, and compared to *RECK* sequences from a variety of other organisms to confirm its identity. *Xenopus laevis RECK* had a full-length coding region of 2, 532 base pairs and a protein length of 843 amino acids.

To confirm that the newly cloned *X. laevis RECK* sequence contains all of the hallmark domains, sequence analyses were performed comparing RECK protein sequences of vertebrate and invertebrate species to the *H. sapiens* sequence. Alignment scores yielded the following order of similarity to *H. sapiens*: *C. familiaris, M. musculus, G. gallus, X. laevis, O. niloticus,* and *D. melanogaster* (Table 3). Compared to human, the most highly similar RECK protein sequence is dog, at 95%, and the least similar RECK protein sequence is fruit fly, at 31% (Table 3). Known domains of RECK were then analyzed to determine conservation of RECK proteins among vertebrate and invertebrate species.

Table 3.

Full length RECK protein amino acid sequence similarity scores as ranked with *H. sapiens*

Species	Percent Similarity	Accession Number
Homo sapiens	100%	NP_066934
Canis lupis familiaris	95%	NP_001002985
Mus musculus	93%	NP_057887
Gallus gallus	84%	XP_418897
Xenopus laevis	78%	N/A
Oreochromis niloticus	58%	XP_003457562
Drosophila melanogaster	31%	NP_648733

The middle portion of all well characterized RECK proteins contain three Kazal motifs (serine-protease inhibitor-like domains). Compared to human, the Kazal motif domains of vertebrate RECK sequences share high similarity, whereas invertebrate RECK sequences share low similarity (Table 4). Moreover, Kazal motif 1 and 3 of *X. laevis* RECK sequence share 85% and 86% similarity to human, respectively (Table 4). Kazal motif 2 is less similar among species, and as such, Kazal motif 2 of *X. laevis* RECK sequence shares only 65% similarity to human RECK (Table 4).

The middle portion of RECK also contains two epidermal growth factor (EGF)-like repeats. Compared to human, the EGF-like repeats of vertebrate RECK sequences share high similarity (>62%), whereas invertebrate RECK sequences share low similarity (<32%) (Table 5). The first EGF-like repeat of *X. laevis* RECK sequence shares 87% similarity to *H. sapiens* RECK, whereas the second EGF-like repeat of *X. laevis* RECK shares only 65% similarity with *H. sapiens* RECK (Table 5).

3.2 Clustalw2 analysis of *X. laevis* RECK revealed sequence conservation among vertebrate species

To further identify similarities of RECK proteins among a variety of species, a phylogram was generated using Clustalw2 software (Figure 5). RECK protein sequences used for this analysis were from the following species: *H. sapiens, C. familiaris, M. musculus, C. familiaris, G. gallus, X. laevis, O. niloticus,* and *D. melanogaster*. Sequence analysis placed *X. laevis* RECK as more closely related to chicken, mouse, dog, and human, than to fish or fly.

Table 4.

Conservation of the amino acid sequence of the three Kazal motif domains of RECK as ranked with *H. sapiens* (percent similarity)

Species	Kazal motif 1	Kazal motif 2	Kazal motif 3
Homo sapiens	100%	100%	100%
Canis lupis familiaris	98%	86%	94%
Mus musculus	93%	88%	94%
Gallus gallus	93%	77%	89%
Xenopus laevis	85%	65%	86%
Oreochromis niloticus	65%	47%	69%
Drosophila melanogaster	50%	16%	51%

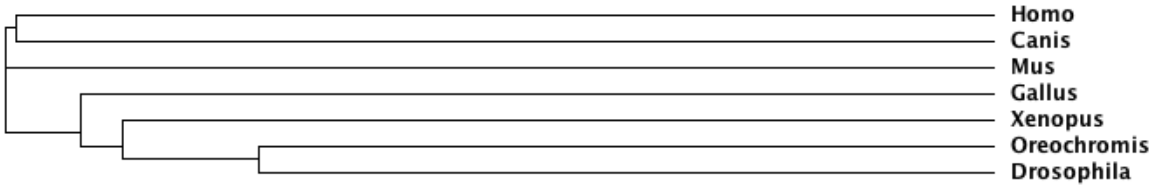
Table 5.

Conservation of the amino acid sequence of the two epidermal growth factor (EGF)-like repeats of RECK as ranked with *H. sapiens* (percent similarity)

Species	EGF-like repeat 1	EGF-like repeat 2
Homo sapiens	100%	100%
Canis lupis familiaris	94%	94%
Mus musculus	90%	88%
Gallus gallus	81%	82%
Xenopus laevis	87%	65%
Oreochromis niloticus	65%	62%
Drosophila melanogaster	32%	21%

Figure 5.

Sequence analysis of RECK. Sequence comparison showing relative similarity of RECK protein was performed using Clustalw2 analysis software at the European Bioinformatics Institute site at www.ebi.ac.uk/Tools/msa/clustalw2/ using default settings. The following are proteins and accession numbers used for this analysis: *Homo sapiens* (NP_066934), *Canis lupis familiaris* (NP_001002985), *Mus musculus* (NP_057887), *Gallus gallus* (XP_418897), *Oreochromis niloticus* (XP_003457562), *Drosophila melanogaster* (NP_648733).


Homo
Canis
Mus
Gallus
Xenopus
Oreochromis
Drosophila

3.3 RT-PCR analysis of *X. laevis RECK* transcript levels during early development

In order to examine the temporal expression pattern of *RECK* during early development, semi-quantitative PCR was performed. Developmental stages chosen to be analyzed included gastrulation (stage 10), closure of the neural tube (stage 18), further neural elongation (stage 24), and organogenesis (stages 32, 37, and 40). *RECK* mRNA levels were measured relative to *EF1α*, a housekeeping gene that maintains consistent levels throughout development (Rupp and Weintraub, 1991; Monsoro-Burq *et al.*, 2003). *RECK* levels were present at all stages throughout early development (Figure 6). *RECK* transcript levels were low during gastrulation (stage 10) and increased during neurulation (stage 18). During later stages of development (stages 24, 32, 37, and 40), *RECK* transcripts maintained relatively consistent levels.

3.4 RT-PCR analysis of *X. laevis TIMP-2* transcript levels during early development

The temporal expression of *TIMP-2* was also examined during early development to determine if TIMP-2 followed a similar expression pattern to *RECK*. *TIMP-2* mRNA levels were measured relative to *EF1α*. *TIMP-2* transcript levels were not present during gastrulation (stage 10), but gradually increased during neurulation (stage 20) and organogenesis (stage 30-40) (Figure 7).

3.5 Whole mount *in situ* hybridization of *X. laevis RECK* during early development

Semi-quantitative PCR analysis revealed that *RECK* transcript levels do not peak until neurulation. As such, a DIG-labeled anti-sense RNA probe was used to detect *RECK* transcripts in developing embryos at stages 23, 28, 35, and 38 of development. A

Figure 6.

Temporal expression analysis of *RECK* mRNA levels during early *X. laevis* development. RT-PCR analysis of *RECK* transcripts measured relative to *EF1α* was performed on embryos at stages 10 through to 40. *RECK* expression is low early on in development during gastrulation (stage 10) and increases during neurulation (stage 18 to 24) and into organogenesis (stages 32 to 40). Standard error bars are representative of three replications.

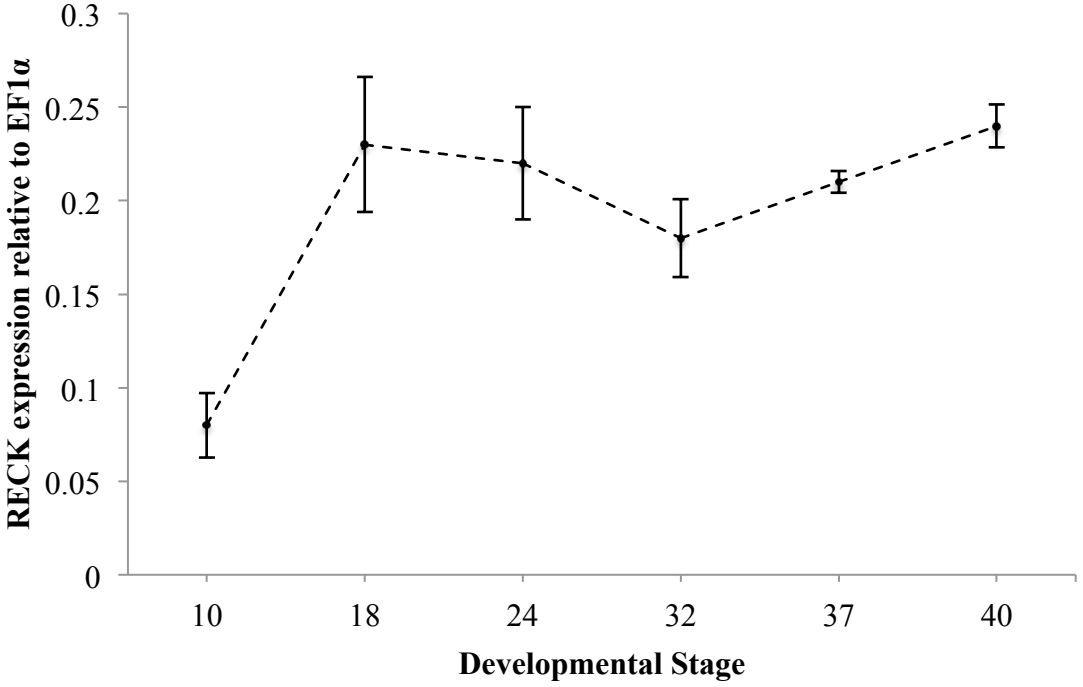
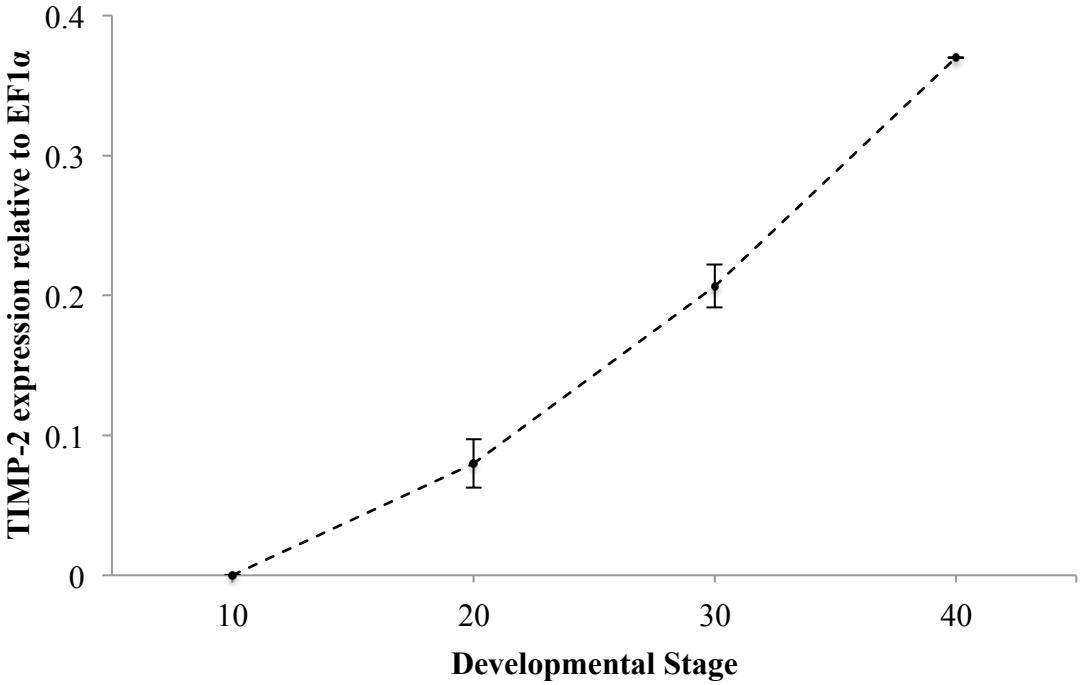

RECK expression relative to EF1α
0.3
0.25
0.2
0.15
0.1
0.05
0
10
18
24
32
37
40
Developmental Stage

Figure 7.

Temporal expression analysis of *TIMP-2* mRNA levels during early *X. laevis* development. RT-PCR analysis of TIMP-2 transcripts measured relative to EF1α was performed on embryos at stages 10, 20, 30, and 40. TIMP-2 transcripts are not detected until neurulation (stage 20). TIMP-2 transcript levels slowly increase throughout organogenesis, peaking at stage 40. Standard error bars are representative of three replications.


0.4
0.3
0.2
0.1
0
TIMP-2 expression relative to EF1α
10
20
30
40
Developmental Stage

DIG-labeled anti-sense RNA probe for *cardiac troponin 1*, a heart specific marker, was used as a positive control (Figure 8A), and no probe was used as a negative control (Figure 8B). Results showed differential expression of *RECK* transcripts throughout development. *RECK* expression was present in dorsal neural structures, including the neural tube, notochord, and brain at the late tailbud stage (stage 28) (Figure 9B,E). However, during later stages of organogenesis (stage 35), *RECK* expression was not detectable in the notochord but remained localized to the neural tube and brain (Figure 9C). Throughout development, *RECK* expression was strongly present in anterior structures such as the eye, branchial arches, and otic placode (Figure 9A-D). Also of importance was the appearance of *RECK* transcripts on the ventral side of the embryos in the blood precursors at stage 35 (Figure 9C).

3.6 Immunohistochemistry of RECK proteins in early *X. laevis* embryos

To further examine the expression pattern of RECK during early *X. laevis* development, immunohistochemistry was performed on sections of early tailbud embryos to detect RECK proteins. RECK proteins were localized to punctate structures along the somites and along the dorsal side of the notochord in early tailbud embryos (Figure 10A). Punctate staining was also present in tissues in the head, including regions of the brain, eye, and cement gland (Figure 10B). RECK proteins were not detected along the neural tube of the embryo. β-catenin was used as a positive control (Figure 10C).

3.7 RT-PCR analysis of the effect of LiCl treatment on the expression level of *RECK* in *X. laevis* embryos

Whole mount *in situ* hybridization of *RECK* transcripts during early *X. laevis*

Figure 8.

Whole mount *in situ* hybridization of *cardiac troponin 1* in early *X. laevis* embryos. Whole mount *in situ* hybridization of stage 28 embryos with antisense Digoxigenin-labeled RNA probes against *cardiac troponin 1* (A) was performed to establish specificity in the experiment. The experiment was also performed without a probe as a negative control (stage 35 shown) (B). Abbreviations: H=heart. Scale bar: 1 mm.

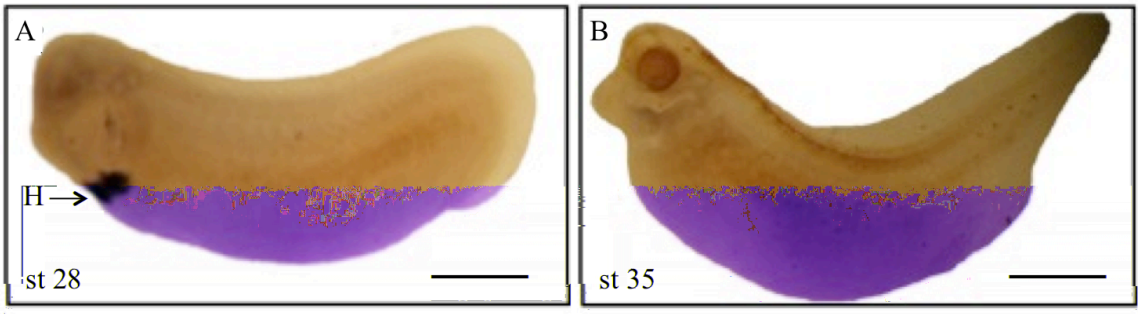

A
H
st 28
B
st 35

Figure 9.

Spatial expression pattern of *RECK* mRNA during early *X. laevis* development. Whole mount *in situ* hybridization was performed on embryos at stages 23 (A), 28 (B), 35 (C), and 38 (D) to determine spatial expression of *RECK* using antisense Digoxigenin-labeled RNA probes against the amplicons of the Kazal motif domains (Table 1). Panel E is a magnified view of a cross section indicated in panel B. *RECK* expression is strongly present in anterior structures such as the eye and otic placode throughout early development (A-D). At late tailbud stage (B), *RECK* expression is present on dorsal neural structures such as the neural tube and notochord. However, by stage 35 (C), *RECK* expression disappears from the notochord and appears in the blood precursors on the ventral side of the embryo. Abbreviations: BA=branchial arches, BP=blood precursors, E=eye, N=notochord, NT=neural tube, OP=otic placode. Scale bar: 1 mm.

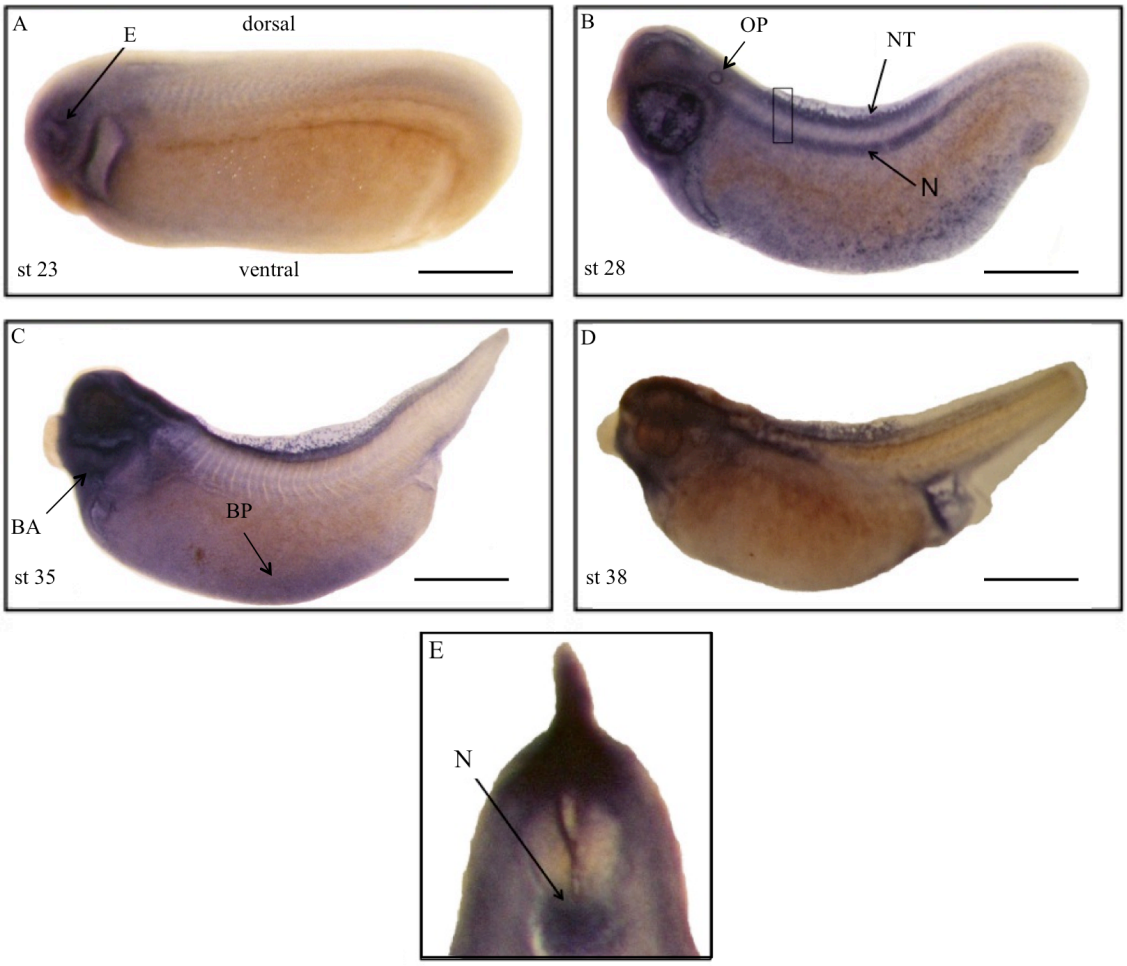
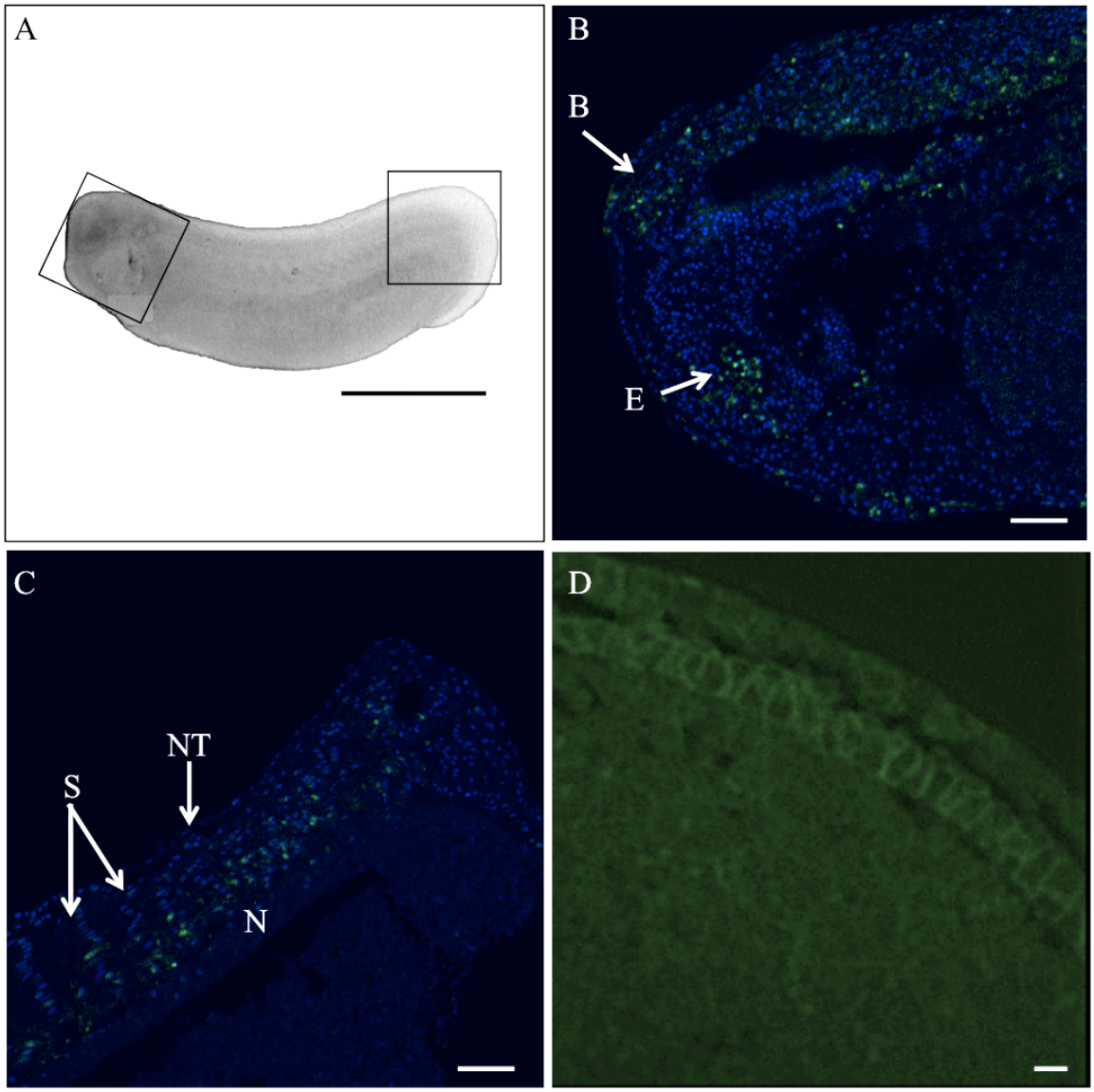

A
E
dorsal
st 23
ventral
B
OP
NT
N
st 28
C
BA
BP
st 35
D
st 38
E
N

Figure 10.

Immunohistochemistry of RECK proteins in early *X. laevis* tailbud embryos (stage 25). Immunohistochemistry was performed using anti-RECK antibody on sections of early tailbud embryos. Sections were counterstained with DAPI (blue). (A) Squares on representative embryo represent the locations of histological sections in B and C. Scale bar: 1mm. (B) RECK protein (green) is localized to anterior head regions such as the brain and eye. Scale bar: 100μm. (C) RECK protein is also localized to the notochord and ventral somites. Scale bar: 100μm. (D) Anti-β-catenin antibody was used as a positive control. Scale bar: 10μm. Abbreviations: B=brain, S=somites, N=notochord, NT=neural tube.


A
B
B
E
C
NT
S
N
D

development showed localization of *RECK* in dorsal and anterior structures. To determine whether *RECK* levels change when dorso-ventral axis patterning is disrupted, *X. laevis* axis formation was perturbed. Early embryos were treated with lithium chloride to induce dorsalization. Dorsalized embryos with a DAI score of 8 were achieved. Dorsalized embryos completely lacked a trunk, had exaggerated heads and lacked ventral structures (Figure 11A) compared to untreated control embryos (Figure 11B).

RNA was isolated from stages 20, 30, and 40 from LiCl-treated embryos and untreated control embryos and RT-PCR analysis was performed. The expression levels of *chordin*, a well-established dorsal mesodermal marker, were measured to confirm dorsalization of LiCl-treated embryos. *Chordin* transcript levels, measured relative to *EF1α*, were higher in LiCl-treated embryos compared to untreated control embryos at all stages examined (Figure 11C). *RECK* expression levels were also measured relative to *EF1α*. *RECK* levels were unchanged in LiCl-treated embryos relative to control embryos at all stages examined (Figure 11D).

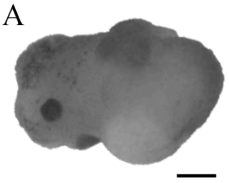
3.8 RT-PCR analysis of the effect of UV treatment on the expression level of *RECK* in *X. laevis* embryos

In dorsalized embryos, *RECK* expression levels did not change, however, I wanted to determine if *RECK* expression levels would increase in ventralized embryos. Prior to completion of the first cell cycle, embryos were exposed to UV light to induce ventralization. Treated embryos were severely ventralized and had a DAI score of 0. Ventralized embryos were largely yolk-containing and completely lacked anterior and dorsal structures, including the head, neural tube, and tail (Figure 12A). Untreated

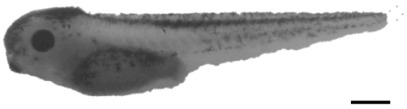
Figure 11.

Semi-quantitative PCR analysis of *chordin* and *RECK* levels in lithium chloride-treated *X. laevis* embryos. LiCl-treated embryos were examined at stages 20, 30, and 40. RT-PCR analysis of chordin and *RECK* transcript levels measured relative to *EF1α* was performed on treated and non-treated embryos. (A) Embryos treated with 0.3M LiCl for 10 minutes were dorsalized and displayed exaggerated heads and severely reduced trunks (stage 40 shown). Scale bar: 1 mm. (B) Embryos that were not treated developed normally (stage 40 shown). Scale bar: 1 mm. (C) *Chordin* transcript levels increased in LiCl-treated embryos compared to control embryos at all stages examined. (D) No change in *RECK* transcript levels were seen at any of the stages examined. Standard error bars are representative of three replications.

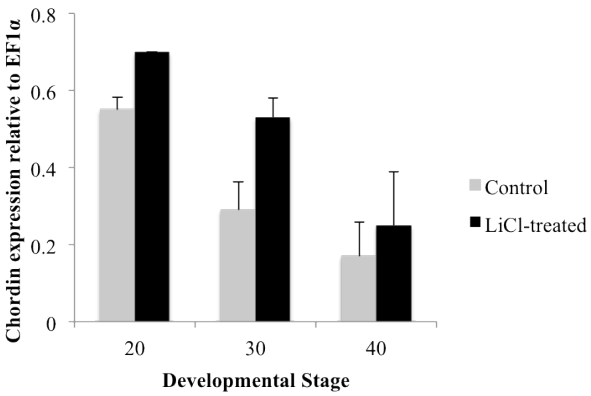
A



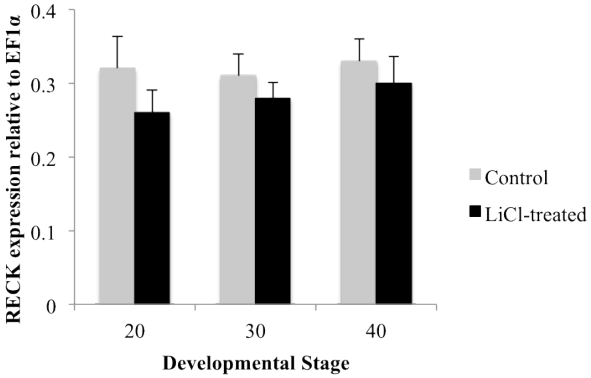
B



C


0.8
0.6
0.4
0.2
0
Chordin expression relative to EF1α
20
30
40
Developmental Stage
Control
LiCl-treated

D


0.4
0.3
0.2
0.1
0
RECK expression relative to EF1α
20
30
40
Developmental Stage
Control
LiCl-treated

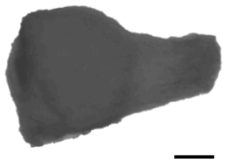
control embryos were maintained under the same conditions and developed normally (Figure 12B).

RNA was isolated from stages 20, 30, and 40 from UV-treated embryos and untreated control embryos and RT-PCR analysis was performed. The expression levels of *chordin* were measured to confirm ventralization of UV-treated embryos. Chordin levels, measured relative to *EF1α*, were lower in UV-treated embryos compared to untreated control embryos at all stages examined (Figure 12C). *RECK* expression levels were measured relative to *EF1α*. *RECK* levels were unchanged in UV-treated embryos compared to control embryos at all stages examined (Figure 12D).

Figure 12.

Semi-quantitative PCR analysis of *chordin* and *RECK* levels in UV-treated *X. laevis* embryos. UV-treated embryos were examined at stages 20, 30, and 40. RT-PCR analysis of chordin and *RECK* transcript levels measured relative to *EF1α* was performed on treated and non-treated embryos. (A) Embryos treated with UVC light for 3 minutes were severely ventralized, containing all yolk and completely lacking anterior and dorsal structures (stage 30 shown). Scale bar: 1 mm. (B) Embryos that were not treated developed normally (stage 30 shown). Scale bar: 1 mm. (C) *Chordin* transcript levels decreased in UV-treated embryos compared to control embryos at all stages examined. (D) No change in *RECK* transcript levels were seen at any of the stages examined. Standard error bars are representative of three replications.

A



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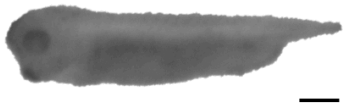
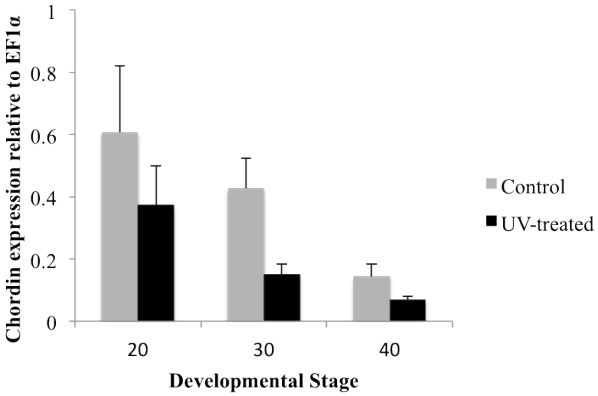
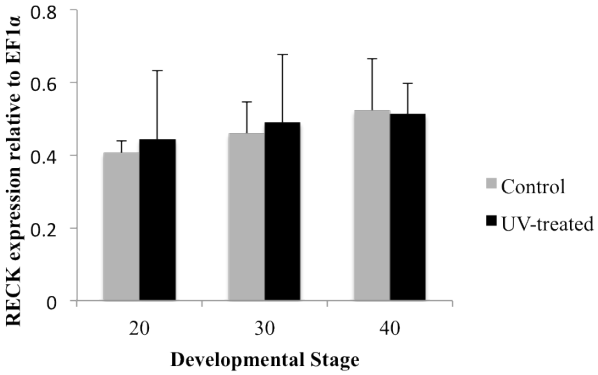


C


Chordin expression relative to EF1α
1
0.8
0.6
0.4
0.2
0
20
30
40
Developmental Stage
Control
UV-treated

D


RECK expression relative to EF1α
0.8
0.6
0.4
0.2
0
20
30
40
Developmental Stage
Control
UV-treated

CHAPTER 4

DISCUSSION

4.1 Overview

In this study, I cloned and characterized the mature coding region of *X. laevis RECK* and examined the expression pattern of *RECK* during early embryonic development. Sequence analysis indicated that *X. laevis RECK* is evolutionarily conserved among vertebrates. As discussed below, when the expression pattern of RECK was analyzed during early *X. laevis* development, RECK expression was low early on in development during gastrulation and increased during neurulation and into organogenesis. Furthermore, *RECK* transcripts were localized to anterior and dorsal structures in early embryos.

4.2 *X. laevis* RECK is highly similar with vertebrate RECK

Proteins that share high sequence similarities among different species are usually considered evolutionarily significant and thus serve important functions (Giudice 2001). In this study, I chose to clone the mature *X. laevis RECK* sequence lacking the N- and C-terminal domains, as these domains were found to be poorly conserved even within vertebrates. Since these domains are not directly related to RECK function, their sequences could be variable between species. Indeed, sequence analysis confirms this (see Appendix I). While the N- and C-terminal domains are not highly conserved, the important functional domains should be. As such, I focused on sequencing known functional domains of the RECK protein.

The newly cloned *X. laevis* RECK protein sequence was compared to RECK protein sequences from a variety of other species. Sequence analysis revealed that *X. laevis* RECK is highly similar to *H. sapiens* RECK. Although a phylogenetic analysis of

RECK proteins has been performed in the past (Knorr *et al.*, 2009), *X. laevis* RECK was not included in this comparison. When RECK amino acid sequences from both vertebrates and invertebrates were aligned, the most related RECK sequences were human, dog, and mouse, and the least related RECK sequence was fruit fly. *Xenopus laevis* RECK was found to share high similarity to mammalian and non-mammalian vertebrate species. This result suggests that RECK proteins serve a similar role among different species.

Hallmark domains of RECK were further analyzed to determine conservation within RECK proteins that may reveal important functions. For the most part, all domains of RECK were highly conserved among vertebrates. As comparisons were made while phylogenetic distance increased, conservation of domains decreased; however, this pattern is often seen with other proteins and genes.

The three Kazal motifs of RECK were found to display an above average level of similarity between vertebrates and invertebrates compared to other domains. As previously mentioned, the Kazal motifs are protease inhibitor-like domains, and as such, presumably play an important role in inhibiting MMPs. Interestingly, Chang *et al.* (2008) determined that the Kazal motifs of RECK are important in inhibiting MMPs. They generated recombinant proteins containing Kazal motifs 2 and 3 and found that these recombinant proteins inhibited MMPs when transfected into mouse lung cancer cells. Thus, the high conservation of Kazal motifs domains among vertebrates and invertebrates suggests a conservation of RECK function as an MMP inhibitor.

Overall, *X. laevis* RECK contains all of the hallmark motifs that are characteristic to RECK proteins. The high overall amino acid sequence similarity between *X. laevis* and other vertebrate RECK proteins suggests a conservation of function. Indeed, studies have shown a correlation of RECK function between species. Oh *et al.* (2001) and Prendergast *et al.* (2012) discovered impaired vascular development in *RECK*-deficient mouse and zebrafish embryos, respectively.

4.3 *RECK* mRNA levels correlate with ECM remodeling events during *X. laevis* development

RECK mRNA expression was examined during various stages of early *X. laevis* development. This was done to identify possible correlations between *RECK* expression, known functions related to regulation of ECM remodeling and cell migration, and developmental events known to involve extensive ECM remodeling. When temporal data were examined, interestingly, results showed that *RECK* expression was low during gastrulation, a developmental event that requires extensive ECM remodeling (Urry *et al.*, 1998; Pickard and Damjanovski, 2004). This result suggests that RECK does not play a role in regulating ECM remodeling during gastrulation. Likely other MMP inhibitors, such as TIMP-3, which is known to be present at this time (Pickard and Damjanovski, 2004), tightly regulate large-scale ECM turnover events that are occurring during development.

RECK expression was more readily detectable at stage 18, during which time the neural tube closed, and *RECK* levels remained relatively elevated all the way through to stage 40, during which time axis elongation was occurring and organs began to develop.

Neurulation and organogenesis also require extensive ECM remodeling (Urry *et al.*, 1998; Pickard and Damjanovski, 2004), but here remodeling becomes increasingly more localized and refined. Damjanovski *et al.* (2000) revealed distinct spatial distribution patterns of several *MMP* mRNAs throughout the developing embryo. Perhaps *RECK* is present at high levels during neurulation and organogenesis to regulate MMP activity, ensuring proper components of the ECM are being remodeled to allow for controlled local cell migration. For instance, Prendergast *et al.* (2012) revealed that *RECK* expression was required for the differentiation of neural crest cells in zebrafish embryos, a process that occurs following closure of the neural tube. Moreover, Oh *et al.* (2001) discovered the importance of RECK during vascular development in mouse embryos.

Although research is limited, a previous study by Knorr *et al.* (2009) revealed high levels of *RECK* mRNA expression during early development of the red flour beetle, *Tribolium castaneum*. *RECK* levels were high throughout the pupal stage, during which time the beetle undergoes metamorphosis. This event requires precise local ECM remodeling, so perhaps *RECK* is expressed during these stages to regulate MMP activity and thus ECM remodeling.

Interestingly, TIMP-2, a well-characterized MMP inhibitor, also follows a similar expression pattern during early *X. laevis* development. *TIMP-2* transcripts were not present during gastrulation; however, *TIMP-2* transcripts increased during neurulation and organogenesis. Since RECK and TIMP-2 share similar mRNA expression patterns, perhaps their role in development is linked. Indeed, TIMP-2 indirectly upregulates RECK by binding its C-terminal domain to α3β1 integrins on the surface of human endothelial cells (Oh *et al.*, 2004).

The expression pattern of RECK during early development revealed that RECK is expressed at increased levels during given stages of development that require extensive ECM remodeling (such as neurulation and organogenesis). Perhaps RECK function at these stages is regulated and mitigated by other inhibitors such that RECK plays an accessory role with respect to TIMP-2. Moreover, perhaps RECK plays a role in protecting structures from undergoing excessive ECM remodeling by regulating MMP activity and that this role increases as differentiation is occurring. For example, Chandana *et al.* (2010) suggested that RECK may play a protective role during vascular development in mice. When histological sections of head and liver of RECK-deficient mice were examined, blood vessels appeared abnormally large and irregularly shaped. These results suggested that RECK protects blood vessels from fusing together and instead facilitates branching (Chandana *et al.*, 2010).

4.4 *RECK* transcripts are localized to anterior and dorsal structures in *X. laevis* embryos

To further examine *RECK* expression during early *X. laevis* development, whole mount *in situ* hybridization was performed on early embryos. *RECK* mRNA was abundantly expressed in anterior and dorsal structures of the embryo, including the neural tube, notochord, and brain. Prendergast *et al.* (2012) also performed whole mount *in situ* hybridization to detect *RECK* transcripts in developing zebrafish embryos. The pattern of localization of RECK in *X. laevis* embryos was similar to that observed in zebrafish embryos.

A number of *MMP* mRNAs are localized along the dorsal axis of *X. laevis* embryos (Damjanovski *et al.*, 2000). Perhaps RECK is present in precise dorsal structures to control MMP activity and thus regulate proper ECM remodeling associated with specific cellular events such as cell migration. For example, Prendergast *et al.* (2012) detected *RECK* mRNA in neural crest cells of early zebrafish embryos. Their results suggested that expression of *RECK* in neural crest cells was necessary to regulate proper formation of DRG, a process that requires cell migration.

Interestingly, *RECK* was transiently expressed in the notochord, as expression disappeared late in development during which extensive cell death events are occurring in the notochord. *Type II collagen* mRNA surrounds the notochord (Bieker and Yazdani-Buicky, 1992), and for axis elongation to occur as the embryo grows, these many large structural proteins such as collagen have to be degraded. The expression pattern of *RECK* suggests that RECK is involved in early specific developmental events such as neurulation and is likely less involved in more large scale ECM remodeling events such as those that occur during axis elongation. Therefore, it is not surprising that *RECK* is absent in late notochord apoptosis and axis elongation events. In fact, Prendergast *et al.* (2012) also found transient *RECK* expression on the dorsal side of zebrafish embryos. Furthermore, as previously mentioned, RECK is required for the differentiation of neural crest cells in zebrafish embryos (Prendergast *et al.*, 2012), a process that occurs early in development following neural tube closure.

RECK expression was also abundant in the head region, such as the eye and otic vesicles, throughout the stages examined. Formation of these organs requires extensive cell movements and controlled ECM remodeling (Saha *et al.*, 1989; Grainger 1992). Any

disruption in the balance between the levels of MMPs and inhibitors during development can cause developmental defects. For example, overexpression of *TIMP-3* mRNA, known to be expressed in the eye of *X. laevis* embryos, caused abnormalities in eye formation (Pickard and Damjanovski, 2004). Perhaps RECK is present along with other MMP inhibitors during the formation of these organs to ensure proper development.

RECK expression was also abundant in the head region of the late embryo as it is preparing to filter feed. During these stages, extensive but specific ECM remodeling is required for the opening of the mouth, formation of the nasal pitts, and branching of the branchial arches (Nieuwkoop and Faber, 1956). All of these processes occur in different regions of the head and require localized ECM remodeling events, thus, there are no global ECM degradation events occurring at this time. Perhaps RECK regulates localized cell migration events rather than global events. Furthermore, MMPs are expressed in a tissue specific manner during development. For example, *MMP-11* mRNA is abundantly present in the branchial arches in *X. laevis* embryos, and less so in the eyes and otic vesicles, whereas *MMP-18* mRNA expression is abundant in the ventral head region in *X. laevis* embryos in an area where the opening to the mouth is forming (Damjanovski *et al.*, 2000). Perhaps *RECK* expression is linked to the type of MMP that is expressed in a given tissue. For example, in mice, *RECK* is expressed in lung tissue along with high levels of *MMP-2* and *MT1-MMP* transcripts (Nuttall *et al.*, 2004). Since RECK has previously been shown to inhibit MMP-2 and MT1-MMP activity, perhaps its role in lung tissue is to control ECM remodeling by regulating MMP-2 and MT1-MMP.

Interestingly, *RECK* expression was also present on the ventral side of the embryo during later stages of development when blood precursors form. Perhaps RECK is

involved in blood differentiation on the ventral side of the embryo. As well, the presence of *RECK* in the branchial arches suggests that RECK may play a role in the branching of blood vessels. For example, as previously mentioned, Oh *et al.* (2001) revealed that *RECK*-deficient mouse embryos displayed enlarged blood vessels and abdominal hemorrhaging.

Also of importance was the absence of *RECK* expression in specific tissues of the developing embryo such as the heart and intestine. While the formation of these tissues also requires specific and extensive ECM remodeling, the absence of *RECK* suggests that RECK is not required to regulate MMP activity in these tissues. As such, other MMP inhibitors, such as TIMPs, may be expressed in these organs to tightly regulate MMP activity and ensure proper development. As well, the development of heart and intestine organs involves looping mechanisms, while vascular development, such as the formation of gills, involves branching mechanisms. Perhaps RECK plays an important role in regulating ECM remodeling events that are linked to branching mechanisms. In fact, in mouse embryos, *RECK* transcripts were most abundantly expressed in lung, in which development is characterized by branching of the bronchioles (Nutall *et al.*, 2004).

4.5 RECK protein localization in early *X. laevis* tailbud embryos

To further analyze RECK, localization of RECK proteins was examined in early tailbud embryos (stage 25) by immunohistochemistry. The presence of RECK proteins in the notochord is in accordance with *in situ* data and further indicates a need for RECK in the differentiation of neural structures at early tailbud stages. However, contrary to *in situ* data, RECK proteins were not detected along the neural tube of early tailbud

embryos. Although *RECK* transcripts appeared to localize in the neural tube throughout the developing embryo, perhaps RECK is regulated primarily at the protein level. Thus, at this stage, ECM remodeling events that require *RECK* expression have already passed with respect to neural development.

RECK proteins were also detected in the eye and brain regions in early tailbud embryos. These results further support *in situ* data and suggest the importance of *RECK* expression in these developing tissues to regulate ECM remodeling.

4.6 *RECK* transcript levels are unchanged in dorsalized and ventralized *X. laevis* embryos

To establish that *RECK* mRNA was localized primarily to dorsal and anterior structures in tailbud embryos, embryo development was perturbed with LiCl and UV treatment. Contrary to my prediction, *RECK* mRNA levels did not change following dorsalization or ventralization of embryos. These results could be due to the fact that *RECK* mRNA appeared on ventral structures of the developing embryo as well, although expression appeared to be more abundant on the dorsal side of the embryo. *RECK* expression may change between tissues, where there is large-scale decreases of *RECK* in one area of the embryo and increases in another, however, analysis of total embryonic *RECK* levels do not reflect such changes. Thus, total *RECK* RNA levels remain relatively constant while relative dorsal and ventral levels change. As such, when axis formation was perturbed, *RECK* mRNA levels appeared relatively unchanged. Another possibility could be that RECK is regulated at the protein level, and thus changes in dorso-ventral axis patterning alters RECK protein levels. For instance, RECK has been

shown to be regulated at the post-translational level through glycosylation of asparagine sites (Simizu *et al.*, 2005).

4.7 Conclusions

1) *X. laevis* RECK protein is highly similar to RECK proteins from other species of vertebrates
2) *X. laevis RECK* mRNA levels correlate with specific late ECM remodeling events during development.
3) *X. laevis RECK* localizes to dorsal neural structures in the developing embryo.
4) Axis perturbation of early embryos does not affect global *RECK* mRNA expression levels in the whole embryo.

4.8 General summary and future perspectives

In the present study, *X. laevis* RECK was cloned and its expression profile was examined during early *X. laevis* development. *Xenopus laevis* RECK was found to share high similarity to human RECK. Further sequence analysis revealed that RECK domains were highly conserved among vertebrates, suggesting that RECK proteins serve similar functions in different species. Expression analysis of *RECK* during early *X. laevis* development revealed that *RECK* is present during given ECM remodeling events, such as neurulation and organogenesis. Its downregulation during other important events, such as gastrulation and axis elongation, indicate the importance of regulating RECK during early *X. laevis* development. Axis perturbation in early embryos did not affect *RECK* mRNA levels, suggesting that *RECK* expression is tissue specific. Altogether, this study was the first detailed comprehensive characterization of RECK during early vertebrate

development and helped elucidate the role RECK plays in mediating ECM turnover during development.

Future work analyzing RECK protein expression throughout *X. laevis* development would further identify the role RECK plays during embryonic development. Moreover, overexpression and knockdown experiments of *RECK* in *X. laevis* embryos would further elucidate RECK function during early development and may reveal what types of ECM remodeling events RECK regulates. As MMP activity is high during development, *X. laevis* is an ideal model for studying the *in vivo* interaction of RECK and MMPs. As such, it would also be interesting to measure aberrant MMP activity following overexpression or knockdown of RECK.

Overall, this research allows for a better understanding into how RECK functions during development, and may improve the understanding of its role in pathogenesis.

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APPENDIX I

Conservation of the amino acid sequence of the N-terminal hydrophobic region (signal peptide) of RECK as ranked with *H. sapiens*

Species	Percent Similarity	Accession Number
Homo sapiens	100%	NP_066934
Canis lupis familiaris	88%	NP_001002985
Mus musculus	81%	NP_057887
Gallus gallus	12%	XP_418897
Xenopus laevis	N/A	N/A
Oreochromis niloticus	27%	XP_003457562
Drosophila melanogaster	8%	NP_648733

Conservation of the amino acid sequence of the C-terminal hydrophobic region (glycosylphosphatidylinositol anchoring signal) of RECK as ranked with *H. sapiens*

Species	Percent Similarity	Accession Number
Homo sapiens	100%	NP_066934
Canis lupis familiaris	59%	NP_001002985
Mus musculus	59%	NP_057887
Gallus gallus	21%	XP_418897
Xenopus laevis	N/A	N/A
Oreochromis niloticus	14%	XP_003457562
Drosophila melanogaster	10%	NP_648733

APPENDIX II

Predicted *Xenopus laevis* RECK Protein Sequence

M C R D V C E Q I L S S K S E S R I K H L L L R A P D Y C P T S M I D V W T C I
N S S L P G V S K K S E G W V G L G C C E L A I A V E C R R A C K Q A S S Q N
D I S K S C R K Q Y E T A L I S C I N R N E M G S V C C S Y A G R H T N C R E Y
C Q A I F R T D S S P G P S Q I K A V E N F C A S I S P P L V Q C V N N Y T Q S Y
P M R N P V D S L Y C C D R A E D P Q C Q S A C K R I L M S K K T E P E I V D S
L S E G C T K P L P Q D P L W Q C F L E S S R T V H S G V N I V P P P S A G L D
G A E L H C C S K A N S S N C R D L C T K L Y S T S W G N T Q V W Q E F E F A
C E Y N P L E A P M L T C L A D V R E P C Q L G C R N L T F C T N F N N R P T E
L F R S C N V Q S D Q G A M N D M K L W E K G S I K M P F M N I P V L D I K K
C H P E M W K A I A C S L Q I K P C H S K S R G S I I C K T D C V E I L T K C G D
H S R F P E S H T A E S I C E L L S P S D E N E D C I P L D T Y L R S S P L D N A I
E E V T H P C N P N P C P A N H L C E V N R K G C L P G E P C L P Y F C S Q G C
K L G E T S D F L V R H G V L I Q M P S G S V G C Y K I C T C G Q S G T L E N C
L D M Q C V D L H K S C L V G G Q R K N H G E S F K V D C N I C S C V A G T L
R C S N H Q C P H S E E D R R M F T G L P C N C E D Q F V P V C G Q N G R T Y
P S A C I A R C V G L L D H Q F E F G L C S S V C N P N P C S R N G I P K R K V
C L T S Y E K F G C A Q Y E C I P R H L K C E H S R D P M C D T E N V E H I N L
C T L Y Q R G R L L S Y K G S C Q P F C K S A E P V C G H N G E T Y P N V C S
A Y S D R V A V D Y Y G H C Q D V G I F S D Q G L H N E C L S I Q C P A I P V T
V C K P I I P P G A C C P L C A G V L R I L F D K E K L D T F A T A T K N T P I T
V M D I L Q K I R Q H V S V P Q C D V F G Y L S M E S D I I I L I V P V D S P P K
S N H

APPENDIX III

Multiple sequence alignment using ClustalW2 of RECK protein sequences from various species*

**Homo sapiens* (NP_066934), *Canis lupis familiaris* (NP_001002985), *Mus musculus* (NP_057887), *Gallus gallus* (XP_418897), *Xenopus laevis, Oreochromis niloticus* (XP_003457562), and *Drosophila melanogaster* (NP_648733).

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Homo          ------------------------------------------------------------
Canis         ------------------------------------------------------------
Mus           ------------------------------------------------------------
Gallus        ------------------------------------------------------------
Xenopus       ------------------------------------------------------------
Oreochromis   ----------------------------------------------------MENMLSLLGP 10
Drosophila    MRLSGWEILLFLLPFGVVSAVRQLEENQSIKNDHHHSVERQVRRRNNKSNASVDHHRHAP 60


Homo          ---------------MATVRASLRGALLLLLAVAGVAEVAGGLAPGSAGALCCNHSKDNQ 45
Canis         ---------------MAAVPASPRGALLLLLAVAGVAEVAGGLAPGSAGALCCNHSKDNQ 45
Mus           ---------------MASVRASPRSALLLLLAAAGVAEVTGGLAPGSAGAVCCNHSKDNQ 45
Gallus        ------------------------------------MTEVAD-LNHRTKCLSCCYHAKDNL 24
Xenopus       ------------------------------------------------------------
Oreochromis   DDGGFLFAHLFLRQLPAAVRAVLANSPLLPAKDYRFLAEEADRILLASHPSCCHHAAEFS 70
Drosophila    SSGSKKNSRSHSVSLDSDDYDWLDADTSEAIDSSEVTAVESIRSPGESYDIFTCCNQVFG 120


Homo          MCRDVCEQIFSSKSES-RLKHLLQRAPDYCPETMVEIWNCMNSSLPGVFKKSDGWVGLGC 104
Canis         MCHDVCEQIFSSKSES-RLKHLLQRAPDYCPETMVEIWSCMNSSLPGVFKKSDGWVGLGC 104
Mus           MCRDVCEQIFSSKSES-RLKHLLQRAPDYCPETMVEIWSCMNSSLPGVFKKSDGWVGLGC 104
Gallus        MCRDVCEQILSSKSDS-RLKHLLQRAPEYCPESMGEVWGCINSSLPGVLKKSDGWVGLGC 83
Xenopus       MCRDVCEQILSSKSES-RIKHLLLRAPDYCPTSMIDVWTCINSSLPGVSKKSEGWVGLGC 59
Oreochromis   PCREACDQLTTIKSES-RLKHLLQRLPGYCSESMNELWMCINSTLPGVSRKSEGWVGLGC 129
Drosophila    SCRTACENLSLVEFATGTGGDNRDELHRYCQLHQVEFWTCVNQTFDAITRGAD-WSGRRC 179
               *: .*:::   :  :    .   .   **     :.* *:*.:: .: : :: * *  *

Homo          CELAIALECRQACKQASSKNDISKVCRKEYENALFSCISRNEMGSVCCSYAGHHTNCREY 164
Canis         CELAITLECRQACKQASSKNDISKACRKEYENALFSCISRNEMGSVCCSYAGHHTNCREY 164
Mus           CELAIGLECRQACKQASSKNDISKVCRKEYENALFSCISRNEMGSVCCSYAGHHTNCREF 164
Gallus        CELAIAVECRQACKQASSKNDILKVCRKEYENALFSCINRNEMGSICCSYAGHHTNCREY 143
Xenopus       CELAIAVECRRACKQASSQNDISKSCRKQYETALISCINRNEMGSVCCSYAGRHTNCREY 119
Oreochromis   CELAITTECRKECKQASSKNDITKVCKKTTENSLYSCITKNEMGSTCCSYAGRHTTCREY 189
Drosophila    CQFGVLPHCRNVCATSTRSP--VQNCRRSDEQTLYDCLERQEAADQCCGQA-RTSECLEA 236
              *::.:  .**. *  :: .    : *::  * :* .*: ::* .. **. * : : * *

Homo          CQAIFRTDSSPGPSQIKAVENYCASISPQLIHCVNNYTQSYPMRNPTDSLYCCDRAEDHA 224
Canis         CQAIFRTDSSPGPSQIKAVENYCASISPQLIHCVNNYTQSYPMRNPTDSLYCCDRAEDHA 224
Mus           CQAIFRTDSSPGPSQIKAVENYCASISPQLIHCVNNYTQSYPMRNPTDSLYCCDRAEDHA 224
Gallus        CQAIFRTDSSPGPSQIKAVENYCASISPQLIHCVNNYTQSYPMRNPTDSLYCCDRAEDYA 203
Xenopus       CQAIFRTDSSPGPSQIKAVENFCASISPPLVQCVNNYTQSYPMRNPVDSLYCCDRAEDPQ 179
Oreochromis   CQAIFRTDSTPTVSQISAVKDYCQTHSAQLIHCVNNFTKSYPIHSPVDSLYCCDRAATH- 248
Drosophila    CRAVFEPANDN--HDHVDINGACGDRNADVIQCVNNHTDMTPLANAEQYIPCCEYSSKEH 294
              *:*:*.. .     :   ::. *   .. :::****.*.  *: .. : : **: :
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Homo          CQNACKRILMS----KKTEMEIVDGLIEGCKTQPLPQDPLWQCFLESSQSVHPGVTV--- 277
Canis         CQNACKRILMS----KKTEMEIVDGLIEGCKTQPLPQDPLWQCFLESSQSVHPGVTL--- 277
Mus           CQNACKRILMS----KKTEMEIVDGLIEGCKTQPLPQDPLWQCFLESSQSVHPGVTV--- 277
Gallus        CQTACKRILMS----MKTELEIVDGLIEGCKTMPLPQDPLWQCFLESSRSVHPGVTV--- 256
Xenopus       CQSACKRILMS----KKTEPEIVDSLSEGC-TKPLPQDPLWQCFLESSRTVHSGVNI--- 231
Oreochromis   CQMACRQILRT----MSTEHEIMEGLIKECGSQPLPQEPMWQCFLSSVQ--TPSITP--- 299
Drosophila    CRHTCRNLLHQNATLMERSEVIFGRLEAAGCGSPLPQLPFWQCFLTVTGKLYVPGGSGRA 354
               *: :*:.:*       . .  *.  *       **** *:*****

Homo          ----------------HPPPSTGLDGAKLHCCSKANTSTCRELCTKLYSMSWGNTQSWQE 321
Canis         ----------------HPPPSTGLDGAKLHCCSKANTSTCRELCTKLYSMSWGNTQSWQE 321
Mus           ----------------HPPPSTGLDGAKLHCCSKANTSTCRELCTKLYSMSWGNTQSWQE 321
Gallus        ----------------HPPPSTGLDGAKLHCCSKANSSTCRELCTKLYSTSWGSSQSWQE 300
Xenopus       ----------------VPPPSAGLDGAELHCCSKANSSNCRDLCTKLYSTSWGNTQVWQE 275
Oreochromis   ----------------EDNLPAKMDCAKLHCCSKANTSLCRDMCLEISSN-WG-SQTWQD 341
Drosophila    GGSLAQGRISGQGGSLAEPNHLGMDAAKRQCCEQASSHKCRRLCNQIFTSNWWDAR--SS 412
                              :* *: :**.:*.:  ** :* :: :  *  ::  ..

Homo          FDRFCEYNPVEVSMLTCLADVREPCQLGCRNLTYCTNFNNRPTELFRSCNAQSDQGAMND 381
Canis         FDRFCEYNPVEVSMLTCLADVREPCQLGCRNLTYCTNFNNRPTELFRSCNAQSDQGAMND 381
Mus           FDRICEYNPVEVSMLTCLADVREPCQLGCTNLTYCTNFNNRPTELFRSCTAQSDQGAMSD 381
Gallus        FDRFCEYNAVEVSMLTCLADVREPCQLGCRNLSYCTNFNNRPTELFRSCNSQSDQGAMND 360
Xenopus       FEFACEYNPLEAPMLTCLADVREPCQLGCRNLTFCTNFNNRPTELFRSCNVQSDQGAMND 335
Oreochromis   FDQLCEYNPVETELINCLADVREPCQLGCKDLTYCTNFNNRPTELFRSCNIQSDQGAMND 401
Drosophila    FENECMDQPGERELRRCIESVDAPCELGCQGLSFCSNFNNRPTELFRSCTPEHDAAARED 472
              *:  *  :. *  :  *: .*  **:*** .*::*:**************. : * .* .*

Homo          MKLWE-KGSIKMPFINIPVLDIKKCQPEMWKAIACSLQIKPCHSKSRGSIICKSDCVEIL 440
Canis         MKLWE-KGSIKMPFINIPVLDIKKCQPEMWKAIACSLQIKPCHSKSRGSIICKSDCVEIL 440
Mus           MKLWE-KGSIKMPFISIPVLDIKTCQPEMWKAVACSLQIKPCHSKSRGSIICKSDCVEIL 440
Gallus        MKLWE-KGSIKMPFINIPVLDINKCQPEMWKAIACSLQIKPCHSKSRGSIICKSDCVEIL 419
Xenopus       MKLWE-KGSIKMPFMNIPVLDIKKCHPEMWKAIACSLQIKPCHSKSRGSIICKTDCVEIL 394
Oreochromis   IKLWS-NGTIKMPFMNIPVLDIRKCQPHMWKAVACSLQIKPCHSRSRGSVICKSDCVDIL 460
Drosophila    WQLLQQRGTVRVLGQELFIKNTSRCAPDKWRALVCALQLKPCTRVGLFNGICSEDCHELL 532
               :* . .*::::   .: : :   * *. *:*:.*:**:***   .  . **. ** ::*

Homo          KKCGDQNKFPEDHTAESICELLSPTDDLKN---CIPLDTYLRPSTLG-NIVEEVTHPCNP 496
Canis         KKCGDQNKFPEDHTAESICELLSPTDDLEN---CIPLDTYLRPSTLG-NIVEEVTHPCNP 496
Mus           KKCGDQNKFPEEHTAESICEFLSPADDLES---CIPLDTYLRPSALG-NIIEEVTHPCNP 496
Gallus        KKCGDHNKFPEGHTAESICELLSPTDDLEN---CIPLDTYLSPSSLG-NIVEDVTHPCNP 475
Xenopus       TKCGDHSRFPESHTAESICELLSPSDENED---CIPLDTYLRSSPLD-NAIEEVTHPCNP 450
Oreochromis   TQCGDTKRFHEGQTPERICELLSPIDDPEH---CIPLYKYLTPSSLGTNTVEEVIHPCNP 517
Drosophila    DECLDWT--LQHQRPKDICSRLKEKDDDDSPLPCISLESYLKPGDASPEEFQGITSPCAQ 590
               :* * .   : : .: **. *.  *: .    **.* .** ..  . : .: :  **

Homo          NPCPANELCEVNRKGCPSGDPCLPYFCVQGCKLGEASDFIVRQGTLIQVPSS-------- 548
Canis         NPCPANELCEVNRKGCLSGDPCLPYSCVQGCKLGEASDFIVRQGTLIQVPSS-------- 548
Mus           NPCPANELCEVNRKGCPSADPCLPYSCVQGCKLGEASDFIVRQGTLIQVPSS-------- 548
Gallus        NPCAANQLCEVNRKGCQSGELCLPYLCVPGCKLGEASDFIVRQGTLIQVPSS-------- 527
Xenopus       NPCPANHLCEVNRKGCLPGEPCLPYFCSQGCKLGETSDFLVRHGVLIQMPS--------- 501
Oreochromis   NPCPSNHICQVNRKGCLDELNCQPYLCVPGCKMGEASEFLVQQDARIQVPTR-------- 569
Drosophila    KPCNGSEVCILQRGGNQG------YSCIPGCNLGQDSKLFVPFGSYVRLGKSNLHKKLEV 644
              :** ...:* ::* *         * *  **::*: *.::*  .  ::: .

Homo          -AGEVGCYKICSCGQSGLLENCMEMHCIDLQKSCIVGGKRKSHGTSFSIDCNVCSCFAGN 607
Canis         -AGEVGCYKICSCGQSGLLENCMEMHCIDLQKSCIVGGKRKSHGTSFNIDCNICSCFAGN 607
Mus           -AGEVGCYKICSCGQSGLLENCMEMHCIDLQKSCIVGGKRKSHGTSFTIDCNVCSCFAGN 607
Gallus        -AGDVGCYKICTCGHTGLLENCVEMHCVDLQKSCIVGGQKKSHGTSFNIDCNVCSCFAGN 586
Xenopus       --GSVGCYKICTCGQSGTLENCLDMQCVDLHKSCLVGGQRKNHGESFKVDCNICSCVAGT 559
Oreochromis   -TDPTVCFEVCSCGPSGRLENCVEMPCMDTSKPCIVGGQRKSHGTSFKTDCHNCYCFAGE 628
Drosophila    GQFPLAEHIVCSCGLQGRLEQCQPLPSYMHAHCTLPGARSYRHGSSFYLECNLCSCFAGE 704
                     . :*:**  * **:*  : .    :  : *.:   ** **  :*: * *.**
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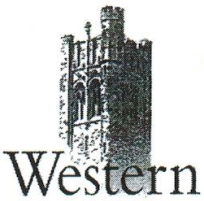
```
Homo           LVCSTRLCLSEHSSEDDRRTFTGLPCNCADQFVPVCGQNGRTYPSACIARCVGLQDHQFE 667
Canis          LVCSTRLCLSEHSSEDDRRTFTGLPCNCADQFVPVCGQNGRTYPSACIARCVGLQDHQFE 667
Mus            LVCSTRLCLSEHSSDDDRRTFTGLPCNCADQFVPVCAQNGRTYPSACIARCVGLQDHQFE 667
Gallus         LICSTRQCLTEHSSEDERQKFTGLPCNCVDQFVPVCGQNGRTYPSACIARCVGLQDNQFE 646
Xenopus        LRCSNHQCP---HSEEDRRMFTGLPCNCEDQFVPVCGQNGRTYPSACIARCVGLLDHQFE 616
Oreochromis    TVCSTKECLSSGYTDDSHRHFTGLPCSCQDRFVPVCASNGRTYPSACVARCMGFKDSQFV 688
Drosophila     ITCTKQQCRLPGFVDSG---YTSLPCNCPAHYVPVCGSNGNTYPSACVAKCH-LPEGDYV 760
                 *:.: *      :.    :*.***.*  ::****..**.******:*:*  : : ::

Homo           FGSCMSKDPCNPNP---CQKNQRCIPKPQVCLTTFDKFGCSQYECVPRQLACDQV-QDPV 723
Canis          FGSCISKDPCNPNP---CPKNQRCIPKPQVCLTTFDKFGCNQYECLPRQLTCDQV-RDPV 723
Mus            FGPCISKNPCNPNL---CPKSQRCVPKPQVCLTTFDKFGCSQYECVPRQLTCDQA-RDPV 723
Gallus         FGSCISKDPCNPNP---CSKNQRCIPKKQVCLTSFGKFECSQHECVPRQLNCDQT-QDPV 702
Xenopus        FGLCSS--VCNPNP---CSRNG--IPKRKVCLTSYEKFGCAQYECIPRHLKCEHS-RDPM 668
Oreochromis    FGPCHLSKPCASKP---CQRNQRCIPKYRVCLS--DVSDCPQYECIGHAATCDKNSEDPA 743
Drosophila     YGACNARNACQAAPPNSCPSGTQCLDSRKVCLASMQRPCLQYVCVNATASNCSTFHQGEV 820
               :* *     * .     *  .   : . :***:                    *.   ..

Homo           CDTDHMEHNNLCTLYQRGKS--LSYKGPCQP--FCRATEPVCGHNGETYSSVCAAYSDRV 779
Canis          CDTNHMEHSNLCTLYQRGQS--LLYKGPCQP--FCRATEPICGHNGETYSSVCAAYSDRV 779
Mus            CDTDHMEHSNLCTLYQRGKS--LSYRGPCQP--FCRAKEPVCGHNGETYSSVCAAYSDRV 779
Gallus         CDTDSVEYSNVCTLYQKGKN--LAYRGPCQP--FCKSVEPVCGHNGETYSSVCAAYSDRV 758
Xenopus        CDTENVEHINLCTLYQRGRL--LSYKGSCQP--FCKSAEPVCGHNGETYPNVCSAYSDRV 724
Oreochromis    CDTEGMDHRSLCHLHQAGKT--LAYMGRCQE--ACRKPKQVCGHNGETYNTVCGAYSDRV 799
Drosophila     CDSQGRTYPNACALLKANPQGQVAYWSACQSSRFNTSPSPVCGINGVTYKSSYAARAEYV 880
               **::   : . * * : .    : * . **        . :** ** ** .  .* :: *

Homo           AVDYYGDCQAVGVLSEHSSVAECASVKCPSLLAAGCKPIIPPGACCPLCAG-MLRVLFDK 838
Canis          AVDYYGPCQAVGVLSEYGSVAECAAVKCPSLSVTECKPIIPPGACCPLCAG-MLRVLFDK 838
Mus            AVDYYGPCQAVGVLSEYSAVAECAAVKCPSLSAIGCKPIIPPGACCPLCAG-MLRVLFDK 838
Gallus         AVDYYGHCQAVGVLSDYGFHTECAFVKCPQLSATGCKPVIAPGACCPLCAG-MLRILYDK 817
Xenopus        AVDYYGHCQDVGIFSDQGLHNECLSIQCPAIPVTVCKPIIPPGACCPLCAG-VLRILFDK 783
Oreochromis    AVDYEAPCRAVGAVSDVAPDSACSLVSCPSLSTPGCHPVTPPGACCPICAS-ILQILWSK 858
Drosophila     LVDYVGRCREVGLLVS-DMGRRCRTVKCPAPVSKHCRLIVPPGACCPLCAGGAFRIIYSR 939
                *** . *: ** . .      *  :.**      *: : .******:**.  ::::::.:

Homo           EKLDTIAK---VTNKKPITVLEILQKIRMHVSVPQCDVFGYFSIESEIVILIIPVD--HY 893
Canis          EKLDTIAK---VTNKKPITVLEILQKIRMHVSVPQCDVFGYFSIESEIVILIIPVD--HY 893
Mus            EKLDTIAK---VTSKKPITVVEILQKVRMHVSVPQCDVFGYLSIESEIVILIIPVD--HY 893
Gallus         DKLDNFAR---VTNKKPITVLDILEKLRLHVSVPQCDVFGYLSIESEIVILIIPVD--QK 872
Xenopus        EKLDTFAT---ATKNTPITVMDILQKIRQHVSVPQCDVFGYLSMESDIIILIVPVD--SP 838
Oreochromis    ERMNTFSK---VWTKQPVTVHDVLQILRPHISVPQCDVFGYLSIDHLLVVIIAPVD--QQ 913
Drosophila     KQFDRAMYGLRAQSSTLLTLQGVLQQLDGLVQVSECQLTGFLTMEVGIFVAIVPSSSIKR 999
               .:::       . ..  :*:  :*: :   :.*.:*:: *:::::  :.: * * .

Homo           PKALQIEACNKEAEKIESLINSDSPTLASHVPLSALIISQVQVSSSVPSAGVRARPSCHS 953
Canis          PKALQIEACIKEAEKIESLINSDSPTLASHVPLSALIISQVQISSSVPSAGIEARALCPS 953
Mus            PKALQIEACNKEAEKIESLINSDSPTLASHVPLSALIISQVQVSSSLPSSAVVGRPLFHS 953
Gallus         PKPLQIEACNKEAEKIESLINSDSPTLASHVPLSALIASQVQVSFSISSPSVKVGPVLHC 932
Xenopus        PKSNH------------------------------------------------------- 843
Oreochromis    PTPLQIEACSKEAEKIDSLINYASPTLVSHVPLSAFLTTEIKTSSIRSSGSTPSSPLTPA 973
Drosophila     PTHLQLEACAREAEKISSLINAQSPRITTNLALSCLTVSHLLEPTPNGATSYGPHAIWIL 1059
               *.  :

Homo           LLLPLSLGLALHLLWTYN 971
Canis          YLLLLSLGPALHMVWIRN 971
Mus            LLLLLSLGLTVHLLWTRP 971
Gallus         LFISFSFTLLKLMDYI-- 948
Xenopus        ------------------
Oreochromis    LCFLLGLLVTPAPVL--- 988
Drosophila     PLLLLVSRVIIA------ 1071
```



Researcher: Dr. S. Damjanovski

Biosafety Approval Number: BIO-UWO-0141

Expiry Date: June 16, 2014

June 21, 2011

Dear Dr. Damjanovski:

Please note your biosafety approval number listed above. This number is very useful to you as a researcher working with biohazards. It is a requirement for your research grants, purchasing of biohazardous materials and Level 2 inspections.

Research Grants:

- This number is required information for any research grants involving biohazards. Please provide this number to Research Services when requested.

Purchasing Materials:

- This number must be included on purchase orders for Level 1 or Level 2 biohazards. When you order biohazardous material, use the on-line purchase ordering system (www.uwo.ca/finance/people/). In the "Comments to Purchasing" tab, include your name as the Researcher and your biosafety approval number.

Annual Inspections:

- If you have a Level 2 laboratory on campus, you are inspected every year. This is your permit number to allow you to work with Level 2 biohazards.

To maintain your Biosafety Approval, you need to:

- Ensure that you update your Biohazardous Agents Registry Form at least every three years, or when there are changes to the biohazards you are working with.
- Ensure that the people working in your laboratory are trained in Biosafety.
- Ensure that your laboratory follows the University of Western Ontario Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories.
- For more information, please see: www.uwo.ca/humanresources/biosafety.

Please let me know if you have questions or comments.

Regards,

Western

2009-044::3:

AUP Number: 2009-044
AUP Title: MMP Activation During Xenopus Development

Approval Date: 05/29/2009

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2009-044 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Jessica Willson

Education

2010-2012	**M.Sc. Candidate,** Department of Biology, Western University, London, ON
2006-2010	**B.Sc. Honors Specialization in Biology,** Western University, London, ON

Honours and Awards

2012	Western Graduate Research Award, Western University, London, ON
2010-2012	Western Graduate Research Scholarship, Western University, London, ON
2008-2010	Dean's Honor List, Western University, London, ON
2006	Western Scholarship of Distinction, Western University, London ON
2006	Ontario Scholar Award, St. Joseph's Catholic High School, St. Thomas, ON

Research Experience

2010-Current **Graduate Research**, Department of Biology, Western University, London, ON

- M.Sc. Thesis: Cloning and analysis of RECK during early *Xenopus laevis* development

2009-2010 **Undergraduate Research**, Department of Biology, Western University, London, ON

- 4999E Thesis: Characterizing cell surface binding of full-length, N- and C-terminal domains of TIMP-2 in *Xenopus laevis* A6 cells

Contributions to Research

Peer Reviewed Publication:

- Shafer, M.E., **Willson, J.A.,** and Damjanovski, S. (2011). Expression analysis of the peroxiredoxin gene family during early development in *Xenopus laevis.* Gene Expression Patterns. 11(8): 511-6.

Poster Presentations:

- **Willson, J.A.** and Damjanovski, S. (2012). Expression analysis of RECK during the early development of *Xenopus laevis*. 25th Annual Western Research Forum. Western University, London, ON.
- Shafer, M.E.R., **Willson, J.A.,** and Damjanovski, S. Characterization of the temporal and spatial expression patterns

of the peroxiredoxin gene family during early development. The Great lakes Mammalian Development Meeting, Toronto, ON, Canada, March 2011.

Teaching Experience

2010-Current **Graduate Teaching Assistant**, Western University, London, ON
- Biology 3338, Developmental Biology
- Biology 2290, Scientific Method in Biology
- Biology 3218G, Biology of the Fungi

2011-Current **Mentor to 4999E Students,** Western University, London, ON

2009-Current **Exam Proctor**, Western University, London, ON